HEPARIN BINDING PROTEINS: SENSORS FOR HEPARIN DETECTION

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CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority of U.S. Provisional Application 60/494,495, filed August 12, 2003, which application is incorporated herein by this reference in its entirety.

I. BACKGROUND OF THE INVENTION

Heparin is a highly heterogeneous glycosaminoglycan (GAG), a family of polysaccharides with alternating uronic acid and aminoglycoside residues that is extracted from mast cells of porcine intestinal mucosa or bovine lung. The chemical modifications, particularly sulfation, lead to pentasaccharide sequences that serve as binding sites for antithrombin III (AT III).[2] In blood, heparin interacts with AT-III, which blocks activation of factor Xa and thereby prevents blood coagulation.[3] The anticoagulant effect of heparin is mediated through this interaction, which markedly accelerates the rate of AT III inhibition of thrombin (factor IIa) and facfor Xa. Heparin detection is very important in the treatment of a number of diseases and therapeutic procedures. There is a need for accurate and simple direct means for detecting heparin. Disclosed are molecules for detecting heparin, and for example, molecules that can quantitate heparin, and methods of using these molecules.

II. SUMMARY

Described herein, are compositions comprising a heparin binding molecules and nucleic acids thereof, as well as methods for making the protein and the nucleic acid, and methods of using the heparin binding protein and nucleic acid thereof.

III. BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification together with the description serve to explain the principles of the invention.

Fig. 1 shows the partial tetrasaccharide structures of HA and heparin.

Fig. 2 shows the schematic preparation of GST-HB1, GST-HB2 and GST-HB3 constructs. Panel A shows RHAMM(518-580). Panel B shows the cloning strategy.

Fig. 3 shows expression and purification of GST-HB proteins. Panel A shows SDS/PAGE of post-sonication supernatant protein expression; boxes show the GST alone and GST-HB fusion proteins. Panel B shows protein purification on GSH-Sepharose beads, following elution of GST and GST-HB proteins with GSH. The lanes are 1,GST; 2, GST-HB1; 3, GST-HB2; 4, GST-HB3.

Fig. 4 shows protein titration for three GST-HB proteins using ELISA with immobilized heparin. Key: diamonds, GST alone; squares, GST-HB1; triangle, GST-HB2; cross, GST-HB3.

Fig. 5 shows competition ELISAs for three GST-HB proteins using immobilized heparin. Competitors, Panel A: HA, CS-A, CS-C, UFH; Panel B, HS, 5 μg/ml and 200 μg/ml; KS, 5 μg/ml and 200 μg/ml. Control: no competitor added.

Fig. 6 shows quantitative competitive ELISAs using immobilized heparin and detection with GST-HB3, A: HA (Mw 190 kDa); B: CS-A; C: CS-C; D: UFH.

Fig. 7 shows measurement of UFH by ELISA with immobilized heparin and GST-HB3 detection. Panel A shows Serial 1:2 dilutions; Panel B shows log-log plot showing linear range over three decades of UFH concentrations.

Fig. 8 shows ELISA quantification of heparin standards in human plasma. Key: squares, UFH; triangles, LMWH.

Fig. 9 shows the plasmid construction for a heparin binding molecule.

Fig. 10 shows a competitive ELISA performed with multiple glycosaminoglycans using biotinylated heparin on a streptavidin-coated plate. Chondroitin sulfate (CS)-A, CS-C, HA, keratan sulfate (KS), heparan sulfate (HS), and unfractionated heparin (UFH) were selected as competitors in a range of 5 μg/ml-200 μg/ml.

Fig. 11 shows a competitive ELISA a clinical assay using both standard well formats. The assay is useful for both the traditional unfractionated heparin (UFH) and the newer low molecular weight heparins (LMWH). Due to the hydrophilic nature of heparin,

streptavidin-coated microtiter plates treated with commercially available biotinylated heparin are used.

Fig. 12 shows a sandwich format ELISA. A "capture protein" is used to coat the wells. HB3-GST is used as the detection probe.

Fig. 13 shows quality control (QC) of a heparin coated surface.

Fig. 14 shows the effect of adding human plasma on heparin ELISA.

Fig. 15 shows the effect of NaCl on heparin ELISA. Key: diamonds, 150 mM, squares, 300 mM, triangles, 500 mM, cross, 750 mM, snowflake, 1000 mM.

Fig. 16 shows analysis of polyelectrolyte theory data for heparin-HB3 binding using a log K_d vs. log[NaCl] plot.

Fig. 17 shows unfractionated heparin was the only glycosaminoglycan that reacted with HBP in specificity studies.

Fig. 18 shows an example of a heparin ELISA, wherein the heparin is bound to the inside of the microplate well.

Fig. 19 shows an example of an ELISA plate setup. These ranges can be used to quantify heparin in a sample.

Fig. 20 shows a competitive ELISA binding reaction. Unknowns and standards were added to the wells, then HB3-HRP was added. The sample was then incubated.

Fig. 21 shows how the assay of Fig. 20 appears after a wash step. TMB was added, then the sample was incubated, stop reagent added, and the plates were read at 450nm.

Fig. 22 shows a low molecular weight heparin (LMWH) ELISA. All major clinical LMWHs are bound.

Fig. 23 shows an unfractionated heparin (UFH) ELISA. All major clinical UFHs are bound.

Fig. 24 shows an ELISA of enoxaparin in plasma. This assay can be used to detect how much heparin is in the plasma of a subject.

Fig. 25 shows an ELISA of UFH in plasma. This assay can be used to detect how much heparin is in the plasma of a subject.

Fig. 26 shows synthetic heparin vs. tinzaparin using a LMWH ELISA. The synthetic heparins are easily measurable using this assay.

Fig. 27 shows an extended range ELISA. Heparin can be detected at less than 0.1 U/ml.

Figure 28 shows the binding of idraparinux using HBMs.

IV. DETAILED DESCRIPTION

Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that the disclosed compositions and methods are not limited to specific synthetic methods, specific compositions, or to particular formulations, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10"as well as "greater than or equal to 10" is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15.

In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

Reference will now be made in detail to the present preferred embodiments of the invention, examples of which are illustrated in the accompanying drawings. Wherever possible, the same reference numbers are used throughout the drawings to refer to the same or like parts.

Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed, that while specific reference to each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular heparin binding molecule (HBM) is disclosed and discussed and a number of modifications that can be made to a number of molecules including the HBM are discussed, specifically contemplated is each and every combination and permutation of the HBM and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

A. Compositions

Disclosed are compositions comprising heparin binding molecules (HBM), wherein the heparin binding molecules are comprised of at least one heparin binding unit. Also disclosed are nucleic acids that encode heparin binding molecules. These compositions aid in the detection of heparin. The compositions are typically composed of a number of parts,

each of which can be a variety of molecules or compositions. Each part of the compositions, how to make them, and how to use them is discussed below.

1. Heparin Binding Molecules

Heparin binding molecules (HBM) can be any molecule that binds heparin. The HBM can be comprised of one or more individual units, called heparin binding units (HBUs). In certain embodiments the molecules bind heparin so that the HBM-heparin complexes can be detected. It is also understood that the HBMs can be linked or combined with any other molecule that may be useful for detection of the HBM, manipulation of the HBM, or, for example, purification of the HBM. In many embodiments the HBM will be a peptide, but as discussed herein the peptides can be modified in many ways to provide a variety of useful characteristics, including increased affinity for heparin, or increased stability, or to, for example, attach the peptide to a solid support. For example, any known heparin binding molecule could be used in conjunction with an HBU or HBM disclosed herein.

a) Peptide HBMs

In certain embodiments the HBM is a peptide based molecule, meaning that one or more of the HBU is a peptide based molecule. In certain embodiments the HBU is comprised of the sequence found in SEQ ID NO: 1, which is two basic amino acids flanking a seven amino acid stretch (hereinafter called BX_7B). The BX_7B molecule is known to be minimally required for binding to hyaluronan[41,60]. This domain has been identified in the N-terminal end of H3P molecules (a precursor to a hyaluronan binding molecule). Furthermore, the BX_7B domain is found within other hyaluronan binding proteins such as aggrecan, CD44, TSG-6, RHAMM, and the link protein. The structures of hyaluronan and heparin GAGs differ substantially, although both are GAGs with alternating uronic acid and glycosamine residues (Figure 1). Hyaluronan is an unsulfated and homogenous glycosaminoglycan (GAG), with a regular repeating disaccharide consisting of alternating glucoronic acid and N-acetylglucosamine residues in alternating β-1,4- and β-1,3 glycosidic linkages. Heparin has 1,4-glycoside linkages and no regular repeat unit; it is heterogenous, having 2 epimeric uronic acids, and both N- and O-sulfation.

One type of protein that contains a HBU is the RHAMM protein (SEQ ID NO: 7). RHAMM belongs to a heterogeneous group of proteins designated hyaladherins, which are linked by their common ability to bind hyaluronan. RHAMM mediates cell migration and proliferation[48], and isoforms can be found in cytoplasm as well as on the surfaces of

activated leukocytes, subconfluent fibroblasts[49, 50] and endothelial cells[51]. RHAMM expression in cell-surface variants promoted tumor progression in selected types of cancer cells[52]. Intracellular RHAMM has been shown to bind to cytoskeletal proteins, to associate with erk kinase, and to mediate the cell cycle through its interaction with $pp60^{v\text{-}src}$[53]. The BX_7B molecule is found within RHAMM. It is understood that in certain embodiments the HBM is not a RHAMM protein, for example, having SEQ ID NO: 7.

The HBU can also be a portion of the RHAMM molecule. For example, RHAMM has been found to contain a 62- amino acid heparin binding domain (HABD) with two base-rich BX_7B motifs, which possesses an overall helix-turn-helix structure (SEQ ID NO: 6, Example 1). This molecule binds with high affinity to heparin as well as to HA. GST fusion proteins containing one, two, or three copies of the RHAMM HABD (HB1, HB2, and HB3, respectively) were cloned, expressed, and purified. The affinity of these proteins for HA and heparin was determined by competitive ELISA. The ELISA employed an immobilized ligand, i.e., biotinylated hyaluronan or biotinylated heparin (HA), bound to a streptavidin-coated microtiter plate. With immobilized HA, each of the three purified fusion proteins showed modest affinity and selectivity for HA. Heparin was over 100-fold more potent as a competitor when compared to free HA as a competitor. Next, an ELISA using biotinylated heparin as the immobilized ligand confirmed affinity for heparin. GST-HB3, in particular, showed a minimum of 100-fold selectivity for heparin over other glycosaminoglycans. GST-HB3 detected calibration standards of both UFH and LMWH that had been added to plasma at very low levels.

Next, an ELISA using biotinylated heparin as the immobilized ligand confirmed that affinity increased with the HABD copy number. The three-copy construct, GST-HB3, showed excellent sensitivity; 0.1 U/ml free heparin was readily measured. Moreover, GST-HB3 showed a minimum 100-fold selectivity for heparin over other glycosaminoglycans. The plot of log K_d vs. log $[Na^+]$ showed between two and three ionic interactions per heparin-HB3 binding based on polyelectrolyte theory (PET). GST-HB3 detected calibration standards of both unfractionated (15 kDa) and low molecular weight (6 kDa) heparin that had been added to human plasma at levels as low as 100 ng/ml. The coefficient of variance for the assay was less than 9% for 6 serial heparin dilutions and was less than 12% for 3 commercial plasma products . These studies demonstrate that GST-HB3 has clinical potential for the quantitative detection of therapeutic heparin levels in plasma, typically ranging between 0.1 U/ml and 2 U/ml.

b) Heparin Binding Unit (HBU)

HBUs are themselves a molecule that have heparin binding activity. These molecules, can be anything that binds heparin, but in many embodiments they will be peptide based molecules. As discussed above, SEQ ID NO:1, BX_7B, is an example of a HBU. Thus, in certain embodiments, a HBM is simply composed of a HBU. However, HBUs are typically linked together to form HBMs, although this is not required for the compositions to display heparin binding activity, as only one HBU is required to form an HBM. An HBM can comprise a single HBU, or an HBU linked to a second HBU, or a first, second, and third HBU all linked together, and so on, for example. There can be at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, or more HBUs linked together. It is understood that that they can be linked in series, i.e. one HBU linked to no more than two other HBUs, or they can be linked in aggregate, i.e., one HBU can be linked to more than two HBUs, such as 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10, or more HBUs.

In addition, the HBUs can be linked via a cleavable bond. Such cleavable linkers allow the individual heparin binding units to be released under reducing conditions, oxidizing conditions, or by hydrolysis of an ester, amide, hydrazide, or similar linkage. Such linkers may include succinates, disulfide-containing chains, and diol-containing chains. It is understood that one HBM can contain different HBUs, linked by different linkers, for example, different cleavable linkers, cleavable linkers and non-cleavable linkers, and so forth. They may also include short peptides with specific targeting sequences for lysosomes and for lysosomal degradation, such as Gly-Phe-Leu-Gly. Other examples include a flexible linker, such as $(GlySer)_9Gly$. Other linkers can be used as well, including peptide linkers, peptide analog linkers, and so forth. The polypeptide linker may be from 1 or 2 amino acids to 100 amino acids in length, or more, with every specific length and combination between 1 and 100 disclosed herein, or between 4 to 50 residues, or optimally between 8 and 30 amino acids in length. Sequences that permit proper folding of the recombinant HBUs expressable in heterologous expression systems could also, for example, use Thr, and/or Ala residues in place of some Ser, Gly residues, and other amino acids may be tolerated. Alternatively, the HBUs may be connected with synthetic, flexible non-peptide linkers, such as polyethylene glycol linkers.

It is understood that when HBUs comprise a protein they can be a recombinant protein, meaning they are made using molecular biology techniques. Thus, a recombinant

protein would be different than a protein that occurs in nature which was isolated, for example.

c) HBM fusion proteins

The HBM can be part of a fusion protein. For example, the HBM can be fused to a glutathione S-transferase (GST) gene. Other fustion partners include but are not limited to His tags (polyhistidine fusion system, vector pET-21d), c-myc tags, FLAG tags, thioredoxin fusions, or maltose binding protein (MBP) fusions, for example. The GST gene fusion system is an integrated system that can be used for the expression, purification and detection of fusion proteins produced in bacterial, yeast, mammalian and insect cells. The sequence encoding the GST protein is incorporated into an expression vector, generally upstream of the multi-cloning site. The sequence encoding the protein of interest is then cloned into the vector. Induction of the vector results in expression of a fusion protein- the protein of interest fused to the GST protein. The fusion protein can then be released from the cells and purified. Purification of the fusion protein is facilitated by the affinity of the GST protein for glutathione residues. Glutathione residues are coupled to a resin and the expressed protein product is brought into contact with the resin. The fusion protein will bind to the glutathione-resin complex and all other non-specific proteins can be washed off. The fusion protein can then be released from the resin using a mild elution buffer which is of low pH. The pH can be from about 0.1 to about 7.0, or from about 1.0 to about 6.0, or from about 2.0 to about 5.0. It is possible to remove the GST from the protein of interest by using a number of different enzymes such as, for example, thrombin and factor X, which cleave specific sites between the GST and the protein of interest. Fusion proteins can also be detected easily, with a number of GST antibodies available on the market.

d) HBM and reporter molecules

The HBM can also comprise reporter molecules. The reporter molecules can be any molecule that allows for detection of the HBM. It is understood that the reporter molecules, can also be linked to the target, of the HBM, such as heparin. The reporter molecules can be anything that allows for detection of the HBM or a molecule bound to the HBM. For example, the reporter molecules can be any chemiluminescent or bioluminescent molecules, but they could also be phosphorescent or radioactive, for example. Those of skill in the art will recognize that there are various reporter molecules and will know how to integrate them for use with the present compositions and methods. Examples of such reporters include, but are not limited to bacterial alkaline phosphatase (BAP) green fluorescent protein (GFP),

beta-glucuronidase (GUS), secreted alkaline phosphatase (SEAP), red fluorescent protein (RFP), horseradish peroxidase conjugation (HRP) and luciferase. Reporter fusion constructs are routinely used in subcellular protein localization, and a user guide to this method recently appeared online in Science's STKE.[45] For example, BAP fusions to SH3 domain binding peptides and PDZ domain binding peptides detect immobilized SH3 domains and PDZ domains in an ELISA-type format.[46] Competition with free peptides demonstrated the specificity of those interactions.

e) HBMs and Capture Tags

In certain aspects HBM fusion proteins can be comprised of capture tags or capture tag receptors. The capture tags can be used to separate molecules which have a capture tag away from molecules which do not. As used herein, a capture tag is any compound that can be associated with a HBM or HBU, or any other composition discussed herein, and which can be used to separate compounds or complexes having the capture tag from those that do not. Preferably, a capture tag is a compound, such as a ligand or hapten, that binds to or interacts with another compound called a capture tag receptor, such as a ligand-binding molecule or an antibody. It is also preferred that such interaction between the capture tag and the capturing component, capture tag receptor, be a specific interaction, such as between a hapten and an antibody or a ligand and a ligand-binding molecule. A capture tag and capture tag receptor combination can be referred to as a capture tag system.

Suitable capture tags include hapten or ligand molecules that can be coupled to the disclosed compositions such as an HBM or HBU. Preferred capture tags, described in the context of nucleic acid probes, have been described by Syvanen *et al.*, *Nucleic Acids Res.*, 14:5037 (1986)), which can be adapted for protein use. Preferred capture tags include biotin, which can be incorporated into nucleic acids or proteins (Langer *et al.*, *Proc. Natl. Acad. Sci. USA* **78**:6633 (1981)) and captured using the capture tag receptors, streptavadin or biotin-specific antibodies. A preferred hapten for use as a capture tag is digoxygenin (Kerkhof, *Anal. Biochem.* **205**:359-364 (1992)). Many compounds for which a specific antibody is known or for which a specific antibody can be generated can be used as capture tags. Such capture tags can be captured by antibodies which recognize the compound. Antibodies useful as capture tags can be obtained commercially or produced using well established methods. For example, Johnstone and Thorpe, *Immunochemistry In Practice* (Blackwell Scientific Publications, Oxford, England, 1987), on pages 30-85, describe general methods useful for producing both polyclonal and monoclonal antibodies. Thus,

any antigen:antibody combination can be used as a capture tag:capture tag receptor, forming a capture tag system.

One type of capture tag is the anti-antibody method. Such anti-antibody antibodies and their use are well known. For example, anti-antibody antibodies that are specific for antibodies of a certain class (for example, IgG, IgM), or antibodies of a certain species (for example, anti-rabbit antibodies) are commonly used to detect or bind other groups of antibodies. Thus, one can have an antibody to the capture tag and then this antibody:capture tag:HBM complex, for example, can then be purified by binding to an antibody for the antibody portion of the complex.

Another type of capture tag is one which can form selectable cleavable covalent bonds with other molecules of choice. For example, a preferred capture tag of this type is one which contains a sulfer atom. An HBU or HBM or any other molecule which is associated with this capture tag can be purified by retention on a thiolpropyl sepharose column. Extensive washing of the column removes unwanted molecules and reduction with β-mercaptoethanol, for example, allows the desired molecules to be collected after purification under relatively gentle conditions (See Lorsch and Szostak, 1994 for a reduction to practice of this type of capture tag).

f) Supports

Capture tags can be associated with the disclosed compositions, such as HBM or HBU, and then the [capure tag:HBM], for example, complex is selectively isolated from the molecules which are not associated with the capture tag. There is then a capture tag receptor (CTR) that can interact with the capture tag complex. In certain embodiments the capture tags or CTRs can be associated with any type of support, such as a solid support. When a CTR is bound to a solid support, capture tag complexes are bound to CTRs of this type they can be effectively purified from the unwanted molecules because the solid support allows for successive washing to remove unbound molecules.

Supports that the CTRs or capture tags can be coupled to can be any solid material to which the CTRs or capture tags can be adhered or coupled. This includes materials such as acrylamide, cellulose, nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, glass, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Supports can have any useful form including thin films or

membranes, beads, bottles, dishes, fibers, woven fibers, shaped polymers, particles and microparticles. Certain forms of supports are plates and beads, and another form are magnetic beads.

Adhering or coupling assay components to a substrate is preferrably accomplished by adhering or coupling CTRs or capture tags to the substrate. The CTRs or capture tags can then mediate adherance of an assay component such as a primer or protein, or for example, an HBM or HBU, by binding to, or interacting with, a capture tag on the component. CTRs or CTs immobilized on a substrate allow capture of the associated molecules, such as an HBM or HBU, on the substrate. Such capture provides a convenient means of washing away reaction components that might interfere with subsequent detection steps. By attaching different CTRs or CTs to different regions of a solid-state detector, different molecules, such as HBMs or HMUs can be captured at different, and therefore diagnostic, locations on the solid-state detector. For example, in a microtiter plate multiplex assay, CTRs or CTs specific for up to 96 different molecules can be immobilized on a microtiter plate, each in a different well. Capture and detection will occur only in those wells corresponding to the specific capture tag system for which the corresponding sample molecules are made.

Methods for immobilization of oligonucleotides to substrates are well established. Oligonucleotides, including oligonucleotide capture docks, can be coupled to substrates using established coupling methods. For example, suitable attachment methods are described by Pease *et al., Proc. Natl. Acad. Sci. USA* **91**(11):5022-5026 (1994), and Khrapko *et al., Mol Biol (Mosk) (USSR)* 25:718-730 (1991). A method for immobilization of 3'-amine oligonucleotides on casein-coated slides is described by Stimpson *et al., Proc. Natl. Acad. Sci. USA* **92**:6379-6383 (1995). A preferred method of attaching oligonucleotides to solid-state substrates is described by Guo *et al., Nucleic Acids Res.* 22:5456-5465 (1994).

Some substrates useful in the disclosed assays have detection antibodies attached to one or more molecules in the assay, such as the capture tag or the molecule attached to the capture tag, or the target sample, the substrate for the molecule attached to the capture tag. Such molecules can be specific for a molecule of interest. Captured molecules of interest can then be detected by binding of a second, reporter molecule, such as an antibody. Such a use of antibodies in a solid-state detector allows assays to be developed for the detection of any molecule for which antibodies can be generated. Methods for immobilizing antibodies

to solid-state substrates are well established. Immobilization can be accomplished by attachment, for example, to aminated surfaces, carboxylated surfaces or hydroxylated surfaces using standard immobilization chemistries. Examples of attachment agents are cyanogen bromide, succinimide, aldehydes, tosyl chloride, avidin-biotin, photocrosslinkable agents, epoxides and maleimides. A preferred attachment agent is glutaraldehyde. These and other attachment agents, as well as methods for their use in attachment, are described in *Protein immobilization: fundamentals and applications*, Richard F. Taylor, ed. (M. Dekker, New York, 1991), Johnstone and Thorpe, *Immunochemistry In Practice* (Blackwell Scientific Publications, Oxford, England, 1987) pages 209-216 and 241-242, and *Immobilized Affinity Ligands*, Craig T. Hermanson *et al.*, eds. (Academic Press, New York, 1992). Antibodies can be attached to a support by chemically cross-linking a free amino group on the antibody to reactive side groups present within the solid-state support. For example, antibodies may be chemically cross-linked to a support that contains free amino or carboxyl groups using glutaraldehyde or carbodiimides as cross-linker agents. In this method, aqueous solutions containing free antibodies are incubated with the solid-state substrate in the presence of glutaraldehyde or carbodiimide. For crosslinking with glutaraldehyde the reactants can be incubated with 2% glutaraldehyde by volume in a buffered solution such as 0.1 M sodium cacodylate at pH 7.4. Other standard immobilization chemistries are known by those of skill in the art.

In addition, non-antibody proteins such as streptavidin, can be linked using similar methods. Many protein and antibody columns are commercially available as well as specifically derivatized supports for conjugation to the CTRs or CTs.

g) Solid-State Samples

Solid-state samples are solid-state substrates or supports to which target molecules or target sequences have been coupled or adhered, for example, through capture tag technology. Target molecules or target sequences are preferably delivered in a target sample or assay sample. One form of solid-state sample is an array sample. An array sample is a solid-state sample to which multiple different target samples or assay samples have been coupled or adhered in an array, grid, or other organized pattern.

Solid-state substrates for use in solid-state samples can include any solid material to which target molecules or target sequences can be coupled or adhered. This includes materials such as acrylamide, cellulose, nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, glass,

polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumarate, collagen, glycosaminoglycans, and polyamino acids. Solid-state substrates can have any useful form including thin films or membranes, beads, bottles, dishes, slides, fibers, woven fibers, shaped polymers, particles and microparticles. Preferred forms for a solid-state substrate are microtiter dishes and glass slides. One form of microtiter dish is the standard 96-well type.

Target molecules and target sequences immobilized on a solid-state substrate allow formation of target-specific molecule combinations localized on the solid-state substrate. Such localization provides a convenient means of washing away reaction components that might interfere with subsequent detection steps, and a convenient way of assaying multiple different samples simultaneously. Diagnostic combinations can be independently formed at each site where a different sample is adhered. For immobilization of target molecules, substrates, to form a solid-state sample, the methods described above for can be used. Where the target molecule is a protein or a polysaccharide, the protein or polysaccharide can be immobilized on a solid-state substrate generally as described above for the immobilization of antibodies.

One form of solid-state substrate is a glass slide to which up to 256 separate target or assay samples have been adhered as an array of small dots. Each dot is preferably from 0.1 to 2.5 mm in diameter, and most preferably around 2.5 mm in diameter. Such microarrays can be fabricated, for example, using the method described by Schena *et al.*, *Science* 270:487-470 (1995). Briefly, microarrays can be fabricated on poly-L-lysine-coated microscope slides (Sigma) with an arraying machine fitted with one printing tip. The tip is loaded with 1μl of a DNA sample (0.5 mg/ml) from, for example, 96-well microtiter plates and deposited ~0.005 μl per slide on multiple slides at the desired spacing. The printed slides can then be rehydrated for 2 hours in a humid chamber, snap-dried at 100°C for 1 minute, rinsed in 0.1% SDS, and treated with 0.05% succinic anhydride prepared in buffer consisting of 50% 1-methyl-2-pyrrolidinone and 50% boric acid. The DNA on the slides can then be denatured in, for example, distilled water for 2 minutes at 90°C immediately before use. Microarray solid-state samples can be scanned with, for example, a laser fluorescent scanner with a computer-controlled XY stage and a microscope objective. A mixed gas, multiline laser allows sequential excitation of multiple fluorophores.

It is understood that the CTs and CTRs and solid supports and solid state components, can be used in any combination. For example, a given assay system, may have

more than one capture tag system employed, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more systems employed. Also, different combinations or solid supports and solid states can be used in any given system. Furthermore, the CTs or CTRs can be usedwith any composition or component or assay or method discussed herein.

h) HBM heparin binding activity

Disclosed are HBMs and variants that bind heparin with a Kd of less than or equal to 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} or 10^{-12}. Furthermore, disclosed are HBMs and variants that bind heparin with an affinity that is at least 2, 4, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 300, or 500 fold greater than the affinity with which it binds another aminoglycosan, such as HA. Furthermore, the HBM can bind molecules other than heparin. For example, HBMs can also bind dextran sulfate, dermatan sulfate, and heparan sulfate. Throughout the specification, the term "heparin" can be used interchangeably with these molecules, and they can be detected and quantified using the same methods disclosed to detect and quantify heparin. Also disclosed are HBMs and variants that have residual heparin binding activity of at least between 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47,. 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 in a residual assay run at 0.1 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47,. 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 125, 150, 200, 250, 300, or 500 minutes, as disclosed herein. The various binding affinities for heparin can be determined as disclosed herein or using any assay for determining binding constants, such as equilibrium dialysis or column chromatography. It is also understood that each individual HBM variant also has a base heparin binding rate which can be determined from the disclosed residual heparin amounts. It is understood that these percentages of base heparin binding rates can be calculated from a base residual heparin amount obtained at any time, which provides data in the analytical range of the assay unless otherwise indicated.

Disclosed are variants of HBMs that have the property of being able to bind heparin. Disclosed are HBMs that bind heparin with at least 5%, 10%, 15%, 20%, 25%, 30% 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and

99%, of the binding activity of a base HBM. It is also understood that each individual HBM variant discussed also has a base heparin binding activity which can be determined from the amount of residual heparin, as disclosed below. It is understood that these percentages of activity can be calculated from a base residual heparin binding activity obtained at any time which provides data in the analytical range of the assay, unless otherwise indicated.

The residual heparin represents the amount of heparin that remains, typically after a 10 minute incubation with heparin and an HBM. The residual heparin is quantified by taking the ratio of the residual heparin after incubation with an HBM to the residual heparin after incubation with buffer. Thus, the lower the residual heparin after incubation with an HBM, the more heparin binding that has taken place by the HBM. The residual heparin can be calculated by subtracting the residual heparin from 100 (100 represents a state of effectively no inhibition). It is understood that if variants of HBMs obtain better binding activity, the timing of the reaction can be decreased, to for example, 9, 8, 7, 6, 5, 4, 3, 2, or 1 minute. For variants of HBMs having less inhibitory activity, the incubation can be increased to, for example, 12, 14, 16, 18, 20, 25, 30, 45, or 60 minutes. One or more assays can be performed with different incubation times to obtain residual heparin amounts that fall between 1 and 100, and, for example, at least two times can be performed for a given HBM so that it can be verified that the assay is being performed in the analytical range. One knows the assay is being performed in the analytical range when two different assays run with two different incubation times produce different residual heparin amounts.

i) Variants

The term "variants" refers to variations in the sequence of either a nucleic acid or a peptide molecule. It is understood that when variants are referred to, the variants designate specific properties dependent on the specific substitutions denoted, however, other substitutions, deletions, and/or insertions, for example, conservative substitutions, insertions, and/or deletions at positions other than the specifically denoted positions are also contemplated provided the variants retain the disclosed activities.

Disclosed are variants that produce HBMs that have the properties disclosed herein. Disclosed are substitutions, wherein the substitutions are made at positions B_1, B_2, X_1, X_2, X_3, X_4, X_5, X_6, or X_7 of the $B_1X_7B_2$ molecule, either alone or in combination. Also disclosed are variants which have 8 amino acids or 6 amino acids between B_1 and B_2. In certain embodiments, the B_1 and B_2 represent basic amino acids and the $X_{1\text{-}7}$ or $X_{1\text{-}6}$ or $X_{1\text{-}8}$ represent any amino acid other than an acidic amino acid as long as one X is a basic amino

acid. Thus, in certain embodiments, X_{1-7} or X_{1-6} or X_{1-8} can be Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr, Cys, Met, Asn, Gln, Arg, Lys, His, Phe, Trp, Pro, but not Asp or Glu, and within the string there must be at least one Arg, Lys, or His. It is understood that every embodiment of the $B_1X_{1-6}B_2$, $B_1X_{1-7}B_2$, or $B_1X_{1-8}B_2$ is specifically disclosed. Applicants have not written each specific species within these sets out, but it is understood that each and every species is specifically disclosed and can be either considered a part of certain embodiments or not a part of certain embodiments. Examples of different $B_1X_{1-6}B_2$, $B_1X_{1-7}B_2$, or $B_1X_{1-8}B_2$ molecules can be found, for example, in Table 1. Other examples can be found by for example performing different Blast analysis relating to the varying HBUs disclosed herein.

Also disclosed are variants with substitutions to the RHAMM (518-580) molecule. Such substitutions can be made throughout the molecule. Yang and Turley (EMBO Journal, 13(2):286-296 (1994) (Which is herein incorporated by reference at least for material related to RHAMM HA binding sequences) provide evidence on HA binding of full-length or soluble RHAMM having only the one BX_7B motif. For example, molecules having substitutions, of any amino acid not exceeding 30% of the amino acids, within the motif and that does not substantially diminish the binding affinity or reduce the heparin selectivity are disclosed. For example, Table 1 provides sequence homology between SEQ ID NO:7, and proteins and peptides which arise in a BLAST search in Genbank. It is understood that certain embodiments do not include the motif BXXBBBXXB and/or BBXXBBBXXBB. (See Sobel et al., J. Biol. Chem., 267:8857-8862 (1992).

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TABLE 1

TBLASTN 2.2.6 [Apr-09-2003]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= (62 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,

GSS, or phase 0, 1 or 2 HTGS sequences)

1,872,777 sequences; 8,818,820,341 total letters

Taxonomy reports

Sequences producing significant alignments:		Score (bits)	E Value
gi|4165078|gb|AF079222.1|AF079222	Mus musculus hyaluronan r...	121	2e-26
gi|1495185|emb|X64550.1|MMRHAMMR	M.musculus mRNA RHAMM	121	2e-26
gi|7305144|ref|NM_013552.1|	Mus musculus hyaluronan mediate...	121	2e-26
gi|3025338|gb|AF031932.1|AF031932	Mus musculus intracellula...	121	2e-26
gi|18204752|gb|BC021427.1|	Mus musculus hyaluronan mediated...	121	2e-26
gi|4580680|gb|AF133037.1|	Rattus norvegicus hyaluronan rece...	119	7e-26
gi|13398479|gb|AF336825.1|	Rattus norvegicus hyaluronan rec...	119	7e-26
gi|6981029|ref|NM_012964.1|	Rattus norvegicus Hyaluronan me...	119	7e-26
gi|1848284|gb|U87983.1|RNU87983	Rattus norvegicus receptor ...	119	7e-26
gi|2959555|gb|U29343.1|HSU29343	Homo sapiens hyaluronan rec...	102	1e-20
gi|7108348|ref|NM_012484.1|	Homo sapiens hyaluronan-mediate...	102	1e-20
gi|3449363|gb|AF032862.1|AF032862	Homo sapiens intracellula...	102	1e-20
gi|23959058|gb|BC033568.1|	Homo sapiens, Similar to hyaluro...	102	1e-20
gi|7108350|ref|NM_012485.1|	Homo sapiens hyaluronan-mediate...	102	1e-20
gi|14582651|gb|AF310973.1|	Ovis aries hyaluronic acid-media...	100	5e-20
gi|20338715|emb|AJ439694.1|BTA439694	Bos taurus partial mRN...	100	5e-20
gi|32766358|gb|BC055178.1|	Danio rerio cDNA clone IMAGE:560...	68	3e-10
gi|19031711|emb|AL646055.10|	Mouse DNA sequence from clone ...	67	7e-10
gi|19387599|gb|AC112205.2|	Homo sapiens chromosome 5 clone ...	66	9e-10
gi|13786277|gb|AC008723.8|AC008723	Homo sapiens chromosome ...	66	9e-10
gi|161411|gb|M58163.1|SUS2B2AA	S.purpuratus open reading frame	66	9e-10
gi|30230907|emb|BX088535.6|	Zebrafish DNA sequence from clo...	50	2e-06
gi|31335230|gb|AY291580.1|	Rattus norvegicus kinesin-like p...	47	7e-04
gi|31795567|ref|NM_181635.2|	Rattus norvegicus kinesin-like...	47	7e-04
gi|31335232|gb|AY291581.1|	Rattus norvegicus kinesin-like p...	47	7e-04
gi|21733494|emb|AL832908.1|HSM804219	Homo sapiens mRNA; cDN...	46	0.001
gi|9910265|ref|NM_020242.1|	Homo sapiens kinesin-like 7 (KN...	46	0.001
gi|9501796|dbj|AB035898.1|	Homo sapiens hklp2 mRNA for kine...	46	0.001
gi|14042773|dbj|AK027816.1|	Homo sapiens cDNA FLJ14910 fis,...	46	0.001
gi|28548928|ref|XM_135231.3|	Mus musculus similar to kinesi...	46	0.001

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gi|1129172|emb|X94082.1|XLKLP2	X.laevis mRNA for KLP2 protein	43	0.008
gi|9887309|gb|AF284333.1|AF284333	Strongylocentrotus purpur...	42	0.023
gi|20336788|gb|AC098649.2|	Homo sapiens chromosome 3 clone ...	37	0.74
gi|22773274|gb|U52111.3|	Homo sapiens chromosome X clone Qc...	33	6.3
gi|1020318|gb|U36341.1|HSU36341	Human Xq28 cosmid, creatine...	33	6.3
gi|26449052|gb|AC133536.2|	Homo sapiens chromosome 16 clone...	33	8.2
gi|29171395|gb|AC138801.2|	Homo sapiens chromosome 16 clone...	33	8.2
gi|1401058|gb|U41302.1|HSU41302	Human chromosome 16 creatin...	33	8.2
gi|29366939|gb|AC010539.9|	Homo sapiens chromosome 16 clone...	33	8.2
gi|29171391|gb|AC136616.4|	Homo sapiens chromosome 16 clone...	33	8.2
gi|29294003|gb|AC140899.3|	Homo sapiens chromosome 16 clone...	33	8.2
gi|29029242|gb|AC133561.4|	Homo sapiens chromosome 16 clone...	33	8.2
gi|29501845|gb|AC009057.10|	Homo sapiens chromosome 16 clon...	33	8.2
gi|25989070|gb|AC136440.3|	Homo sapiens chromosome 16 clone...	33	8.2

Alignments

```
>gi|4165078|gb|AF079222.1|AF079222 Mus musculus hyaluronan receptor
RHAMMV5 mRNA, complete cds
          Length = 2479

 Score =  121 bits (303), Expect = 2e-26
 Identities = 62/62 (100%), Positives = 62/62 (100%)
 Frame = +1

Query: 1    DSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG
Sbjct: 2143 RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 2322

Query: 61   IR 62
             IR
Sbjct: 2323 IR 2328


>gi|1495185|emb|X64550.1|MMRHAMMR M.musculus mRNA RHAMM
          Length = 3167

 Score =  121 bits (303), Expect = 2e-26
 Identities = 62/62 (100%), Positives = 62/62 (100%)
 Frame = +1

Query: 1    RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG
Sbjct: 1807 RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 1986

Query: 61   IR 62
             IR
Sbjct: 1987 IR 1992


>gi|7305144|ref|NM_013552.1| Mus musculus hyaluronan mediated
motility receptor (RHAMM) (Hmmr),
          mRNA
          Length = 3539

 Score =  121 bits (303), Expect = 2e-26
 Identities = 62/62 (100%), Positives = 62/62 (100%)
```

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```
 Frame = +1

Query: 1    RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
            RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG
Sbjct: 2179 RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 2358

Query: 61   IR 62
            IR
Sbjct: 2359 IR 2364


>gi|3025338|gb|AF031932.1|AF031932 Mus musculus intracellular
hyaluronic acid binding protein (IHABP)
          mRNA, complete cds
        Length = 3539

 Score =  121 bits (303), Expect = 2e-26
 Identities = 62/62 (100%), Positives = 62/62 (100%)
 Frame = +1

Query: 1    RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
            RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG
Sbjct: 2179 RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 2358

Query: 61   IR 62
            IR
Sbjct: 2359 IR 2364


>gi|18204752|gb|BC021427.1| Mus musculus hyaluronan mediated
motility receptor (RHAMM), mRNA
          (cDNA clone MGC:29212 IMAGE:5035341), complete cds
        Length = 3695

 Score =  121 bits (303), Expect = 2e-26
 Identities = 62/62 (100%), Positives = 62/62 (100%)
 Frame = +2

Query: 1    RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
            RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG
Sbjct: 2318 RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 2497

Query: 61   IR 62
            IR
Sbjct: 2498 IR 2503


>gi|4580680|gb|AF133037.1| Rattus norvegicus hyaluronan receptor
RHAMM (Rhamm) mRNA, complete
          cds
        Length = 2286

 Score =  119 bits (299), Expect = 7e-26
 Identities = 61/62 (98%), Positives = 61/62 (98%)
 Frame = +1

Query: 1    RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
            RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQL KRKQNELRLQGELDKALG
Sbjct: 1888 RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLAKRKQNELRLQGELDKALG 2067
```

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```
Query: 61    IR 62
             IR
Sbjct: 2068  IR 2073
```

>gi|13398479|gb|AF336825.1| Rattus norvegicus hyaluronan receptor RHAMM mRNA, complete cds
Length = 2286

```
 Score =  119 bits (299), Expect = 7e-26
 Identities = 61/62 (98%), Positives = 61/62 (98%)
 Frame = +1

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQL KRKQNELRLQGELDKALG
Sbjct: 1888  RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLAKRKQNELRLQGELDKALG 2067

Query: 61    IR 62
             IR
Sbjct: 2068  IR 2073
```

>gi|6981029|ref|NM_012964.1| Rattus norvegicus Hyaluronan mediated motility receptor (RHAMM) (Hmmr), mRNA
Length = 2049

```
 Score =  119 bits (299), Expect = 7e-26
 Identities = 61/62 (98%), Positives = 61/62 (98%)
 Frame = +1

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQL KRKQNELRLQGELDKALG
Sbjct: 1525  RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLAKRKQNELRLQGELDKALG 1704

Query: 61    IR 62
             IR
Sbjct: 1705  IR 1710
```

>gi|1848284|gb|U87983.1|RNU87983 Rattus norvegicus receptor for hyaluronan-mediated motility mRNA, complete cds
Length = 2049

```
 Score =  119 bits (299), Expect = 7e-26
 Identities = 61/62 (98%), Positives = 61/62 (98%)
 Frame = +1

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQL KRKQNELRLQGELDKALG
Sbjct: 1525  RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLAKRKQNELRLQGELDKALG 1704

Query: 61    IR 62
             IR
Sbjct: 1705  IR 1710
```

```
>gi|2959555|gb|U29343.1|HSU29343  LUG Homo sapiens hyaluronan receptor
(RHAMM) mRNA, complete cds
          Length = 2756

 Score =  102 bits (254), Expect = 1e-20
 Identities = 51/62 (82%), Positives = 57/62 (91%)
 Frame = +1

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYA+LLGHQNLKQKIKHVVKLKDENSQLKSEVSKLR QL K+KQ+E +LQ EL+K LG
Sbjct: 1927  RDSYAKLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRCQLAKKKQSETKLQEELNKVLG 2106

Query: 61    IR 62
             I+
Sbjct: 2107  IK 2112


>gi|7108348|ref|NM_012484.1|  LUG Homo sapiens hyaluronan-mediated
motility receptor (RHAMM) (HMMR),
            transcript variant 1, mRNA
          Length = 3002

 Score =  102 bits (254), Expect = 1e-20
 Identities = 51/62 (82%), Positives = 57/62 (91%)
 Frame = +1

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYA+LLGHQNLKQKIKHVVKLKDENSQLKSEVSKLR QL K+KQ+E +LQ EL+K LG
Sbjct: 1900  RDSYAKLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRCQLAKKKQSETKLQEELNKVLG 2079

Query: 61    IR 62
             I+
Sbjct: 2080  IK 2085

>gi|3449363|gb|AF032862.1|AF032862  LUG Homo sapiens intracellular
hyaluronic acid binding protein (IHABP)
            mRNA, complete cds
          Length = 3002

 Score =  102 bits (254), Expect = 1e-20
 Identities = 51/62 (82%), Positives = 57/62 (91%)
 Frame = +1

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYA+LLGHQNLKQKIKHVVKLKDENSQLKSEVSKLR QL K+KQ+E +LQ EL+K LG
Sbjct: 1900  RDSYAKLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRCQLAKKKQSETKLQEELNKVLG 2079

Query: 61    IR 62
             I+
Sbjct: 2080  IK 2085


>gi|23959058|gb|BC033568.1|  U Homo sapiens, Similar to hyaluronan-
mediated motility receptor
           (RHAMM), clone IMAGE:4777447, mRNA
          Length = 1856

 Score =  102 bits (254), Expect = 1e-20
 Identities = 51/62 (82%), Positives = 57/62 (91%)
 Frame = +3
```

```
Query: 1    RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
            RDSYA+LLGHQNLKQKIKHVVKLKDENSQLKSEVSKLR QL K+KQ+E +LQ EL+K LG
Sbjct: 735  RDSYAKLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRCQLAKKKQSETKLQEELNKVLG 914

Query: 61   IR 62
            I+
Sbjct: 915  IK 920
```

>gi|7108350|ref|NM_012485.1| LUG Homo sapiens hyaluronan-mediated motility receptor (RHAMM) (HMMR),
transcript variant 2, mRNA
Length = 2957

```
 Score =  102 bits (254), Expect = 1e-20
 Identities = 51/62 (82%), Positives = 57/62 (91%)
 Frame = +1

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             RDSYA+LLGHQNLKQKIKHVVKLKDENSQLKSEVSKLR QL K+KQ+E +LQ EL+K LG
Sbjct: 1855  RDSYAKLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRCQLAKKKQSETKLQEELNKVLG 2034

Query: 61    IR 62
             I+
Sbjct: 2035  IK 2040
```

>gi|14582651|gb|AF310973.1| Ovis aries hyaluronic acid-mediated motility receptor mRNA, partial
cds
Length = 249

```
 Score =  100 bits (248), Expect = 5e-20
 Identities = 50/62 (80%), Positives = 56/62 (90%)
 Frame = +3

Query: 1   RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
           RDSYA+LLGHQNLKQKIKHVVKLKDENS LKSEV KLR+QL KRKQ+E +LQ EL+K LG
Sbjct: 45  RDSYAKLLGHQNLKQKIKHVVKLKDENSNLKSEVLKLRAQLTKRKQSEAKLQEELNKVLG 224

Query: 61  IR 62
           I+
Sbjct: 225 IK 230
```

>gi|20338715|emb|AJ439694.1|BTA439694 U Bos taurus partial mRNA for receptor for hyaluronic acid mediated
motility (rhamm gene)
Length = 249

```
 Score =  100 bits (248), Expect = 5e-20
 Identities = 50/62 (80%), Positives = 56/62 (90%)
 Frame = +3

Query: 1   RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
           RDSYA+LLGHQNLKQKIKHVVKLKDENS LKSEV KLR+QL KRKQ+E +LQ EL+K LG
Sbjct: 45  RDSYAKLLGHQNLKQKIKHVVKLKDENSNLKSEVLKLRAQLTKRKQSEAKLQEELNKVLG 224
```

```
Query: 61   IR 62
            I+
Sbjct: 225  IK 230
```

>gi|32766358|gb|BC055178.1| Danio rerio cDNA clone IMAGE:5604784, partial cds
Length = 1892

```
 Score = 67.8 bits (164), Expect = 3e-10
 Identities = 33/50 (66%), Positives = 42/50 (84%)
 Frame = +3

Query: 3    SYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQ 52
             +YA L+GHQN +QKIKH+VKLK+EN +LK EVSKLRSQ+ K+KQ   RL+
Sbjct: 1152 AYANLMGHQNQRQKIKHMVKLKEENLELKQEVSKLRSQVGKQKQELDRLK 1301
```

>gi|19031711|emb|AL646055.10| Mouse DNA sequence from clone RP23-382C18 on chromosome 11, complete sequence
Length = 193551

```
 Score = 66.6 bits (161), Expect = 7e-10
 Identities = 32/32 (100%), Positives = 32/32 (100%)
 Frame = -2

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKS 32
             RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKS
Sbjct: 79028 RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKS 78933


 Score = 60.8 bits (146), Expect = 4e-08
 Identities = 31/33 (93%), Positives = 31/33 (93%)
 Frame = -2

Query: 30    LKSEVSKLRSQLVKRKQNELRLQGELDKALGIR 62
             L   EVSKLRSQLVKRKQNELRLQGELDKALGIR
Sbjct: 76985 LSQEVSKLRSQLVKRKQNELRLQGELDKALGIR 76887
```

>gi|19387599|gb|AC112205.2| Homo sapiens chromosome 5 clone RP11-80G7, complete sequence
Length = 137376

```
 Score = 66.2 bits (160), Expect = 9e-10
 Identities = 34/43 (79%), Positives = 37/43 (86%)
 Frame = +3

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVK 43
             RDSYA+LLGHQNLKQKIKHVVKLKDENSQLKS V K+     +K
Sbjct: 53877 RDSYAKLLGHQNLKQKIKHVVKLKDENSQLKS-VCKMTFHFIK 54002
```

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```
 Score = 42.0 bits (97), Expect = 0.018
 Identities = 20/30 (66%), Positives = 25/30 (83%)
 Frame = +3

Query: 33     EVSKLRSQLVKRKQNELRLQGELDKALGIR 62
              EVSKLR QL K+KQ+E +LQ EL+K LGI+
Sbjct: 60117 EVSKLRCQLAKKKQSETKLQEELNKVLGIK 60206
```

```
>gi|13786277|gb|AC008723.8|AC008723  Homo sapiens chromosome 5 clone
CTB-95B16, complete sequence
          Length = 109616

 Score = 66.2 bits (160), Expect = 9e-10
 Identities = 34/43 (79%), Positives = 37/43 (86%)
 Frame = +2

Query: 1     RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVK 43
              RDSYA+LLGHQNLKQKIKHVVKLKDENSQLKS V K+    +K
Sbjct: 84377 RDSYAKLLGHQNLKQKIKHVVKLKDENSQLKS-VCKMTFHFIK 84502
```

```
 Score = 42.0 bits (97), Expect = 0.018
 Identities = 20/30 (66%), Positives = 25/30 (83%)
 Frame = +2

Query: 33     EVSKLRSQLVKRKQNELRLQGELDKALGIR 62
              EVSKLR QL K+KQ+E +LQ EL+K LGI+
Sbjct: 90617 EVSKLRCQLAKKKQSETKLQEELNKVLGIK 90706
```

```
>gi|161411|gb|M58163.1|SUS2B2AA  S.purpuratus open reading frame
          Length = 3356

 Score = 66.2 bits (160), Expect = 9e-10
 Identities = 29/59 (49%), Positives = 45/59 (76%)
 Frame = +3

Query: 2    DSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELDKALG 60
             + YA+LLGHQN KQKI H++K+K+KDEN+ LK EV+KLR +  K+ +N   +++ +++K  G
Sbjct: 2058 NDYAKLLGHQNQKQKIHHIMKIKDENASLKKEVTKLREETTKQSRNLRQMKDKVEKMEG 2234
```

```
>gi|30230907|emb|BX088535.6|  Zebrafish DNA sequence from clone DKEY-
18F5 in linkage group 14, complete
          sequence
          Length = 197465

 Score = 49.7 bits (117), Expect(2) = 2e-06
 Identities = 21/30 (70%), Positives = 27/30 (90%)
 Frame = +3

Query: 2      DSYAQLLGHQNLKQKIKHVVKLKDENSQLK 31
               D+YA L+GHQN +QKIKH+VKLK+EN +LK
Sbjct: 109383 DAYANLMGHQNQRQKIKHMVKLKEENLELK 109472
```

```
 Score = 25.0 bits (53), Expect(2) = 2e-06
 Identities = 13/20 (65%), Positives = 16/20 (80%)
 Frame = +1

Query: 33     EVSKLRSQLVKRKQNELRLQ 52
              EVSKLRSQ+ K+KQ   RL+
Sbjct: 109552 EVSKLRSQVGKQKQELDRLK 109611


>gi|31335230|gb|AY291580.1| Rattus norvegicus kinesin-like protein
KIF15 mRNA, complete cds
          Length = 4214

 Score = 46.6 bits (109), Expect = 7e-04
 Identities = 22/43 (51%), Positives = 32/43 (74%)
 Frame = +3

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNE 48
             +L+GHQNL QKI++VV+LK EN +L  E  KLR++ V  K+ +
Sbjct: 4047 KLIGHQNLHQKIQYVVRLKKENIRLAEETEKLRAENVFLKERK 4175


>gi|31795567|ref|NM 181635.2| Rattus norvegicus kinesin-like 7
(Knsl7), mRNA
          Length = 4214

 Score = 46.6 bits (109), Expect = 7e-04
 Identities = 22/43 (51%), Positives = 32/43 (74%)
 Frame = +3

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNE 48
             +L+GHQNL QKI++VV+LK EN +L  E  KLR++ V  K+ +
Sbjct: 4047 KLIGHQNLHQKIQYVVRLKKENIRLAEETEKLRAENVFLKERK 4175


>gi|31335232|gb|AY291581.1| Rattus norvegicus kinesin-like protein
KIF15 mRNA, complete cds
          Length = 4210

 Score = 46.6 bits (109), Expect = 7e-04
 Identities = 22/43 (51%), Positives = 32/43 (74%)
 Frame = +3

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNE 48
             +L+GHQNL QKI++VV+LK EN +L  E  KLR++ V  K+ +
Sbjct: 4047 KLIGHQNLHQKIQYVVRLKKENIRLAEETEKLRAENVFLKERK 4175


>gi|21733494|emb|AL832908.1|HSM804219 Homo sapiens mRNA; cDNA
DKFZp762D1914 (from clone DKFZp762D1914)
          Length = 3696

 Score = 46.2 bits (108), Expect = 0.001
```

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```
 Identities = 22/43 (51%), Positives = 32/43 (74%)
 Frame = +3

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNE 48
            +L+GHQNL QKI++VV+LK EN +L  E  KLR++ V  K+ +
Sbjct: 3012 KLVGHQNLHQKIQYVVRLKKENVRLAEETEKLRAENVFLKEKK 3140
```

```
>gi|9910265|ref|NM_020242.1| LUG Homo sapiens kinesin-like 7 (KNSL7),
mRNA
          Length = 4775

 Score = 46.2 bits (108), Expect = 0.001
 Identities = 22/43 (51%), Positives = 32/43 (74%)
 Frame = +2

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNE 48
            +L+GHQNL QKI++VV+LK EN +L  E  KLR++ V  K+ +
Sbjct: 4097 KLVGHQNLHQKIQYVVRLKKENVRLAEETEKLRAENVFLKEKK 4225
```

```
>gi|9501796|dbj|AB035898.1| LU Homo sapiens hklp2 mRNA for kinesin-like
protein 2, complete cds
          Length = 4775

 Score = 46.2 bits (108), Expect = 0.001
 Identities = 22/43 (51%), Positives = 32/43 (74%)
 Frame = +2

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNE 48
            +L+GHQNL QKI++VV+LK EN +L  E  KLR++ V  K+ +
Sbjct: 4097 KLVGHQNLHQKIQYVVRLKKENVRLAEETEKLRAENVFLKEKK 4225
```

```
>gi|14042773|dbj|AK027816.1| LU Homo sapiens cDNA FLJ14910 fis, clone
PLACE1006368, weakly similar to
               HYALURONAN-MEDIATED MOTILITY RECEPTOR
          Length = 2441

 Score = 46.2 bits (108), Expect = 0.001
 Identities = 22/43 (51%), Positives = 32/43 (74%)
 Frame = +3

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNE 48
            +L+GHQNL QKI++VV+LK EN +L  E  KLR++ V  K+ +
Sbjct: 1779 KLVGHQNLHQKIQYVVRLKKENVRLAEETEKLRAENVFLKEKK 1907
```

```
>gi|28548928|ref|XM_135231.3| L Mus musculus similar to kinesin-like 7;
kinesin-like protein 2
               [Homo sapiens] (LOC235683), mRNA
          Length = 1566

 Score = 46.2 bits (108), Expect = 0.001
 Identities = 23/45 (51%), Positives = 33/45 (73%), Gaps = 2/45 (4%)
 Frame = +3
```

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```
Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQ--LVKRKQNE 48
            +L+GHQNL QKI++VV+LK EN +L  E  KLR++   +K K+ E
Sbjct: 822 KLVGHQNLHQKIQYVVRLKKENIRLTEETEKLRAENLFLKEKKKE 956
```

>gi|1129172|emb|X94082.1|XLKLP2 U X.laevis mRNA for KLP2 protein
Length = 5135

```
 Score = 43.1 bits (100), Expect = 0.008
 Identities = 20/43 (46%), Positives = 32/43 (74%)
 Frame = +1

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNE 48
            ++LGHQN  QKI+++VKLK EN++L  E  KLR + +  K+++
Sbjct: 4159 KILGHQNPNQKIQYLVKLKKENNKLLEEAEKLRIENLFLKESK 4287
```

>gi|9887309|gb|AF284333.1|AF284333 Strongylocentrotus purpuratus kinesin-like protein KRP180 mRNA,
complete cds
Length = 4392

```
 Score = 41.6 bits (96), Expect = 0.023
 Identities = 21/51 (41%), Positives = 31/51 (60%)
 Frame = +1

Query: 6    QLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELD 56
            +L GHQN KQKI H+  +K EN  LK EV  L  QL K + +  +++ + +
Sbjct: 4123 ELGGHQNPKQKIHHLQAVKSENYFLKEEVESLEKQLGKAQSDSEQMKRDYE 4275
```

>gi|20336788|gb|AC098649.2| D Homo sapiens chromosome 3 clone RP11-272D20, complete sequence
Length = 204143

```
 Score = 36.6 bits (83), Expect = 0.74
 Identities = 16/25 (64%), Positives = 22/25 (88%)
 Frame = +3

Query: 6      QLLGHQNLKQKIKHVVKLKDENSQL 30
              +L+GHQNL QKI++VV+LK EN +L
Sbjct: 130584 KLVGHQNLHQKIQYVVRLKKENVRL 130658
```

>gi|22773274|gb|U52111.3| L G D Homo sapiens chromosome X clone Qc-7G6, QLL-F1720, QLL-C1335, Qc-8B7,
Qc-11H12, Qc-7F6, QLL-E153, Qc-10E8, Qc-10B7 map q28,
complete sequence
Length = 247592

```
 Score = 33.5 bits (75), Expect = 6.3
 Identities = 14/30 (46%), Positives = 22/30 (73%)
 Frame = -1

Query: 21   VKLKDENSQLKSEVSKLRSQLVKRKQNELR 50
```

```
               VKL++EN  LK+++ KL+ +L   KQ+E R
Sbjct: 86222 VKLEEEENRSLKADLQKLKDELASTKQSEAR 86133
```

>gi|1020318|gb|U36341.1|HSU36341 LGD Human Xq28 cosmid, creatine transporter (SLC6A8) gene, complete cds, and CDM gene, partial cds

```
          Length = 33023

 Score = 33.5 bits (75), Expect = 6.3
 Identities = 14/30 (46%), Positives = 22/30 (73%)
 Frame = -3

Query: 21    VKLKDENSQLKSEVSKLRSQLVKRKQNELR 50
             VKL++EN  LK+++ KL+ +L   KQ+E R
Sbjct: 20625 VKLEEEENRSLKADLQKLKDELASTKQSEAR 20536
```

>gi|26449052|gb|AC133536.2| D Homo sapiens chromosome 16 clone CTA-17E1, complete sequence

```
          Length = 234771

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = -3

Query: 21    VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
             VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 51964 VKLEEEENRSLKAELQKLKDELASTKQSE 51881
```

>gi|29171395|gb|AC138801.2| D Homo sapiens chromosome 16 clone CTD-3129020, complete sequence

```
          Length = 150183

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = +1

Query: 21    VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
             VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 16675 VKLEEEENRSLKAELQKLKDELASTKQSE 16758
```

>gi|1401058|gb|U41302.1|HSU41302 D Human chromosome 16 creatine transporter (SLC6A8) and (CDM) paralogous genes, complete cds

```
          Length = 32505

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = -1

Query: 21    VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
             VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 19563 VKLEEEENRSLKAELQKLKDELASTKQSE 19480
```

```
>gi|29366939|gb|AC010539.9| Homo sapiens chromosome 16 clone RP11-
373A21, complete sequence
          Length = 101043

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = +2

Query: 21     VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
              VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 62672 VKLEEENRSLKAELQKLKDELASTKQSE 62755


>gi|29171391|gb|AC136616.4| Homo sapiens chromosome 16 clone RP11-
44A7, complete sequence
          Length = 174477

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = +1

Query: 21     VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
              VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 51709 VKLEEENRSLKAELQKLKDELASTKQSE 51792


>gi|29294003|gb|AC140899.3| Homo sapiens chromosome 16 clone RP11-
792K9, complete sequence
          Length = 194490

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = +1

Query: 21      VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
               VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 177682 VKLEEENRSLKAELQKLKDELASTKQSE 177765


>gi|29029242|gb|AC133561.4| Homo sapiens chromosome 16 clone RP11-
598D12, complete sequence
          Length = 169866

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = -2

Query: 21      VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
               VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 102845 VKLEEENRSLKAELQKLKDELASTKQSE 102762
```

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```
>gi|29501845|gb|AC009057.10| Homo sapiens chromosome 16 clone RP11-
274A17, complete sequence
          Length = 170820

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = -2

Query: 21     VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
              VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 156014 VKLEEENRSLKAELQKLKDELASTKQSE 155931


>gi|25989070|gb|AC136440.3| Homo sapiens chromosome 16 clone RP11-
378C4, complete sequence
          Length = 175691

 Score = 33.1 bits (74), Expect = 8.2
 Identities = 14/28 (50%), Positives = 21/28 (75%)
 Frame = -1

Query: 21     VKLKDENSQLKSEVSKLRSQLVKRKQNE 48
              VKL++EN  LK+E+ KL+ +L   KQ+E
Sbjct: 108122 VKLEEENRSLKAELQKLKDELASTKQSE 108039
```

As discussed herein there are numerous variants of the HBM proteins and RHAMM proteins that are known and herein contemplated. In addition, to the known functional strain variants there are derivatives of the HBM and RHAMM proteins which also function in the disclosed methods and compositions. Protein variants and derivatives are well understood to those of skill in the art and in can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Immunogenic fusion protein derivatives, such as those described in the examples, are made by fusing a polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking in vitro or by recombinant cell culture transformed with DNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Tables 2 and 3 and are referred to as conservative substitutions.

TABLE 2:Amino Acid Abbreviations

Amino Acid	Abbreviations	
Alanine	Ala	A
Allosoleucine	AIle	
Arginine	Arg	R
Asparagines	Asn	N
aspartic acid	Asp	D
Cysteine	Cys	C
glutamic acid	Glu	E
Glutamine	Gln	K
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Phenylalanine	Phe	F
Proline	Pro	P
pyroglutamic acid	Glup	
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
tryptophan	Trp	W
Valine	Val	V

TABLE 3:Amino Acid Substitutions

Original Residue	Exemplary Conservative Substitutions, others are known in the art.
Ala	gly. Ser
Ar	glys, gln
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn, lys
Glu	asp
Gly	ala, pro depending upon whether the gly plays a packing role [ala] or a turn role [pro]
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln;
Met	Leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular

Properties, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence. It is also understood that while no amino acid sequence indicates what particular DNA sequence encodes that protein within an organism, where particular variants of a disclosed protein are disclosed herein, the known nucleic acid sequence that encodes that protein in the particular species from which that protein arises is also known and herein disclosed and described.

It is understood that there are numerous amino acid and peptide analogs which can be incorporated into the disclosed compositions. For example, there are numerous D amino acids or amino acids which have a different functional substituent then the amino acids shown in Table 2 and Table 3. The opposite stereo isomers of naturally occurring peptides are disclosed, as well as the stereo isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way (Thorson et al., Methods in Molec. Biol. 77:43-73 (1991), Zoller, Current Opinion in Biotechnology, 3:348-354 (1992); Ibba, Biotechnology & Genetic Enginerring Reviews 13:197-216 (1995), Cahill et al., TIBS, 14(10):400-403 (1989); Benner, TIB Tech, 12:158-163 (1994); Ibba and Hennecke, Bio/technology, 12:678-682 (1994) all of which are herein incorporated by reference at least for material related to amino acid analogs).

Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include CH_2NH--, --CH_2S--, --CH_2--CH_2 --, --CH=CH-- (cis and trans), --$COCH_2$ --, --CH(OH)CH_2--, and --CHH_2SO—(These and others can be found in Spatola, A. F. in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1,

Issue 3, Peptide Backbone Modifications (general review); Morley, Trends Pharm Sci (1980) pp. 463-468; Hudson, D. et al., Int J Pept Prot Res 14:177-185 (1979) (--CH_2NH--, CH_2CH_2--); Hann J. Chem. Soc Perkin Trans. I 307-314 (1982) (--CH--CH--, cis and trans); Almquist et al. J. Med. Chem. 23:1392-1398 (1980) (--$COCH_2$--); Jennings-White et al. Tetrahedron Lett 23:2533 (1982) (--$COCH_2$--); Szelke et al. European Appln, EP 45665 CA (1982): 97:39405 (1982) (--$CH(OH)CH_2$--); Holladay et al. Tetrahedron. Lett 24:4401-4404 (1983) (--$C(OH)CH_2$--); and Hruby Life Sci 31:189-199 (1982) (--CH_2--S--); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is --CH_2NH--. It is understood that peptide analogs can have more than one atom between the bond atoms, such as β-alanine, γ-aminobutyric acid, and the like.

Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be beneficial to constrain peptides into particular conformations. (Rizo and Gierasch Ann. Rev. Biochem. 61:387 (1992), incorporated herein by reference).

(1) Sequence similarities of variants

It is understood that as discussed herein the use of the terms homology and identity mean the same thing as similarity. Thus, for example, if the use of the word homology is used between two non-natural sequences it is understood that this is not necessarily indicating an evolutionary relationship between these two sequences, but rather is looking at the similarity or relatedness between their nucleic acid sequences. Many of the methods for determining homology between two evolutionarily related molecules are routinely applied to any two or more nucleic acids or proteins for the purpose of measuring sequence similarity regardless of whether they are evolutionarily related or not.

1. It is understood that one way to define the variants and derivatives of the disclosed proteins herein is through defining the variants and derivatives in terms of homology/identity to specific known sequences. For example, SEQ ID NO:1 sets forth a

particular sequence of an HBU and SEQ ID NO:7 sets forth a particular sequence of a RHAMM protein. Specifically disclosed are variants of these and other proteins herein disclosed which have at least, 60% or 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least 60%, 70%, 80%, 85%, 90%, 95%, 96%,97%, 98%, or 99% homology to a particular sequence wherein the variants are conservative mutations.

In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein, is through defining the variants and derivatives in terms of homology to specific known sequences. This identity of particular sequences disclosed herein is also discussed elsewhere herein. In general, variants of genes and proteins herein disclosed typically have at least, about 40, 50, 55, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, J. MoL Biol. 48: 443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. U.S.A. 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the

results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above. For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using each of calculation methods (although, in practice, the different calculation methods will often result in different calculated homology percentages).

(2) Hybridization/selective hybridization

The term hybridization typically means a sequence driven interaction between at least two nucleic acid molecules, such as a primer or a probe and a gene. Sequence driven interaction means an interaction that occurs between two nucleotides or nucleotide analogs or nucleotide derivatives in a nucleotide specific manner. For example, G interacting with C or A interacting with T are sequence driven interactions. Typically sequence driven interactions occur on the Watson-Crick face or Hoogsteen face of the nucleotide. The hybridization of two nucleic acids is affected by a number of conditions and parameters known to those of skill in the art. For example, the salt concentrations, pH, and temperature of the reaction all affect whether two nucleic acid molecules will hybridize.

Parameters for selective hybridization between two nucleic acid molecules are well known to those of skill in the art. For example, in some embodiments selective hybridization conditions can be defined as stringent hybridization conditions. For example,

stringency of hybridization is controlled by both temperature and salt concentration of either or both of the hybridization and washing steps. For example, the conditions of hybridization to achieve selective hybridization may involve hybridization in high ionic strength solution (6X SSC or 6X SSPE) at a temperature that is about 12-25°C below the Tm (the melting temperature at which half of the molecules dissociate from their hybridization partners) followed by washing at a combination of temperature and salt concentration chosen so that the washing temperature is about 5°C to 20°C below the Tm. The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to a labeled nucleic acid of interest and then washed under conditions of different stringencies. Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The conditions can be used as described above to achieve stringency, or as is known in the art. (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; Kunkel et al. Methods Enzymol. 1987:154:367, 1987 which is herein incorporated by reference for material at least related to hybridization of nucleic acids). A preferable stringent hybridization condition for a DNA:DNA hybridization can be at about 68°C (in aqueous solution) in 6X SSC or 6X SSPE followed by washing at 68°C. Stringency of hybridization and washing, if desired, can be reduced accordingly as the degree of complementarity desired is decreased, and further, depending upon the G-C or A-T richness of any area wherein variability is searched for. Likewise, stringency of hybridization and washing, if desired, can be increased accordingly as homology desired is increased, and further, depending upon the G-C or A-T richness of any area wherein high homology is desired, all as known in the art.

Another way to define selective hybridization is by looking at the amount (percentage) of one of the nucleic acids bound to the other nucleic acid. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the limiting nucleic acid is bound to the non-limiting nucleic acid. Typically, the non-limiting primer is in for example, 10 or 100 or 1000 fold excess. This type of assay can be performed at under conditions where both the limiting and non-limiting primer are for example, 10 fold or 100 fold or 1000 fold below their k_d, or

where only one of the nucleic acid molecules is 10 fold or 100 fold or 1000 fold or where one or both nucleic acid molecules are above their k_d.

Another way to define selective hybridization is by looking at the percentage of primer that gets enzymatically manipulated under conditions where hybridization is required to promote the desired enzymatic manipulation. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer is enzymatically manipulated under conditions which promote the enzymatic manipulation, for example if the enzymatic manipulation is DNA extension, then selective hybridization conditions would be when at least about 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer molecules are extended. Preferred conditions also include those suggested by the manufacturer or indicated in the art as being appropriate for the enzyme performing the manipulation.

Just as with homology, it is understood that there are a variety of methods herein disclosed for determining the level of hybridization between two nucleic acid molecules. It is understood that these methods and conditions may provide different percentages of hybridization between two nucleic acid molecules, but unless otherwise indicated meeting the parameters of any of the methods would be sufficient. For example if 80% hybridization was required and as long as hybridization occurs within the required parameters in any one of these methods it is considered disclosed herein.

It is understood that those of skill in the art understand that if a composition or method meets any one of these criteria for determining hybridization either collectively or singly it is a composition or method that is disclosed herein.

2. Nucleic Acids

There are a variety of molecules disclosed herein, such as various variant HBMs. It is understood that these peptide based molecules can be encoded by a number of nucleic acids, including for example the nucleic acids that encode, for example, SEQ ID NO:1. It is understood that for example, when a vector is expressed in a cell, that the expressed mRNA will typically be made up of A, C, G, and U.

a) Sequences

There are a variety of sequences related to BX_7B, RHAMM, and subsections of RHAMM such as HABD, which can be found at, for example, in the Genbank database

which can be accessed at www.pubmed.gov. These sequences and others are herein incorporated by reference in their entireties as well as for individual subsequences contained therein. It is also understood that the protein sequences can be found here as well, and are incorporated herein by reference.

One particular sequence set forth in SEQ ID NO: 1 is used herein, as an example, to exemplify the disclosed compositions and methods. Nucleic acids comprising a sequence, wherein the sequence encodes a heparin binding peptide are disclosed. For example, SEQ ID NO: 8 is the nucleic acid molecule corresponding to the peptide sequence of SEQ ID NO: 7. SEQ ID NO: 10 is the nucleic acid molecule corresponding to the peptide sequence of SEQ ID NO: 9. SEQ ID NO: 12 is the nucleic acid molecule corresponding to the peptide sequence of SEQ ID NO: 11.

It is understood that the description related to this sequence is applicable to any sequence related to HBMs unless specifically indicated otherwise. For example, as disclosed above, the HBMs can be fused to various molecules such as fluorescent, chromogenic, or GST molecules. Nucleic acids corresponding to those molecules are also disclosed. The HBM nucleic acid can further comprise a BAP nucleic acid, for instance. The HBM nucleic acid can also further comprise and EGFP nucleic acid. The HBM nucleic acid can also further comprise a bacterial GST nucleic acid.

The nucleic acid can be contained in a vector, such as a plasmid, for example. Examples of such vectors are well known in the art.

Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular sequence to other related sequences (i.e. sequences of an HBM). Primers and/or probes can be designed for any HBM related nucleic acid sequence given the information disclosed herein and known in the art.

b) Primers and probes

Disclosed are compositions including primers and probes, which are capable of interacting with nucleic acids related to HBMs as disclosed herein. In certain embodiments the primers are used to support DNA amplification reactions. Typically, the primers will be capable of being extended in a sequence specific manner. Extension of a primer in a sequence specific manner includes any methods wherein the sequence and/or composition of the nucleic acid molecule to which the primer is hybridized or otherwise associated directs or influences the composition or sequence of the product produced by the extension

of the primer. Extension of the primer in a sequence specific manner therefore includes, but is not limited to, PCR, DNA sequencing, DNA extension, DNA polymerization, RNA transcription, or reverse transcription. Techniques and conditions that amplify the primer in a sequence specific manner are preferred. In certain embodiments the primers are used for the DNA amplification reactions, such as PCR or direct sequencing. It is understood that in certain embodiments the primers can also be extended using non-enzymatic techniques, where for example, the nucleotides or oligonucleotides used to extend the primer are modified such that they will chemically react to extend the primer in a sequence specific manner. Typically the disclosed primers hybridize with the nucleic acids related to HBMs or regions of the nucleic acids related to the HBMs or they hybridize with the complement of the nucleic acids related to the HBMs or complement of a region of the nucleic acids related to the HBM gene. The primers and probes can be any size that meets the requirements of being a primer or probe including, but not limited to 3, 4, or 5 nucleotides long

The size of the primers or probes for interaction with the nucleic acids related to the HBMs in certain embodiments can be any size that supports the desired enzymatic manipulation of the primer, such as DNA amplification or the simple hybridization of the probe or primer. A typical primer or probe for nucleic acids related to the HBMs would be at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3500, or 4000 nucleotides long.

In other embodiments a primer or probe for an HBM can be less than or equal to 6, 7, 8, 9, 10, 11, 12 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3500, or 4000 nucleotides long.

The primers for the nucleic acids related to HBMs typically will be used to produce an amplified DNA product that contains an HBM. In general, typically the size of the product will be such that the size can be accurately determined to within 3, or 2 or 1 nucleotides. In certain embodiments this product is at least 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3500, or 4000 nucleotides long.

In other embodiments the product is less than or equal to 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3500, or 4000 nucleotides long.

Some examples of primers which are useful with the present invention for amplifying the HABD molecule include the following:

SEQ ID NO: 2
5'-CGGGATCCGGTGCTAGCCGTGACTCCTATGCACAGCTCCTTGG-3'
SEQ ID NO: 3
5'-GGAGCGGTCGACACGGATGCCCAGAGCTTTATCTAATTC-3'
SEQ ID NO: 4
5'-GATCCGGTCTCGAGGGAAGTGGTTCTGGAAGTGGTTCAGGTTCGGGTAGCGGATCTGGTTCAGGAAGTGGTT-3'
SEQ ID NO: 5
5'-CTAGAACCACTTCCTGAACCAGATCCGCTACCCGAACCTGAACCACTTCCAGAACCACTTCCCTCGAGACCG-3'

B. Methods of Making

The compositions disclosed herein and the compositions necessary to perform the disclosed methods can be made using any method known to those of skill in the art for that particular reagent or compound unless otherwise specifically noted. It is understood that general molecular biology techniques, such as those disclosed in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold

Spring Harbor, N.Y., 1989) are available for making the disclosed molecules and practicing the disclosed methods unless otherwise noted.

1. Nucleic acid synthesis

For example, the nucleic acids, such as the oligonucleotides to be used as primers, can be made using standard chemical synthesis methods or can be produced using enzymatic methods or any other known method. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 380B). Synthetic methods useful for making oligonucleotides are also described by Ikuta *et al.*, *Ann. Rev. Biochem.* **53**:323-356 (1984), (phosphotriester and phosphite-triester methods), and Narang *et al.*, *Methods Enzymol.*, 65:610-620 (1980), (phosphotriester method). (Peptide nucleic acid molecules) can be made using known methods such as those described by Nielsen *et al.*, *Bioconjug. Chem.* 5:3-7 (1994).

2. Peptide synthesis

One method of producing the disclosed peptides is to link two or more amino acids or peptides together by protein chemistry techniques. For example, amino acids or peptides can be chemically synthesized using currently available laboratory equipment using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc (*tert* -butyloxycarbonoyl) chemistry. (Applied Biosystems, Inc., Foster City, CA). One skilled in the art can readily appreciate that a peptide or polypeptide corresponding to the disclosed proteins, for example, can be synthesized by standard chemical reactions. For example, a peptide or polypeptide can be synthesized and not cleaved from its synthesis resin whereas the other fragment of a peptide or protein can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group which is functionally blocked on the other fragment. By peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, to form a protein , or fragment thereof. (Grant GA (1992) Synthetic Peptides: A User Guide. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) Principles of Peptide Synthesis. Springer-Verlag Inc., NY (which is herein incorporated by reference at least for material related to peptide

synthesis). Alternatively, the peptide or polypeptide is independently synthesized *in vivo* as described herein. Once isolated, these independent peptides or polypeptides may be linked to form a peptide or fragment thereof via similar peptide condensation reactions.

For example, enzymatic ligation of cloned or synthetic peptide segments allow relatively short peptide fragments to be joined to produce larger peptide fragments, polypeptides or whole protein domains (Abrahmsen L et al., Biochemistry, 30:4151 (1991)). Alternatively, native chemical ligation of synthetic peptides can be utilized to synthetically construct large peptides or polypeptides from shorter peptide fragments. This method consists of a two step chemical reaction (Dawson et al. Synthesis of Proteins by Native Chemical Ligation. Science, 266:776-779 (1994)). The first step is the chemoselective reaction of an unprotected synthetic peptide--thioester with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without a change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site (Baggiolini M et al. (1992) FEBS Lett. 307:97-101; Clark-Lewis I et al., J.Biol.Chem., 269:16075 (1994); Clark-Lewis I et al., Biochemistry, 30:3128 (1991); Rajarathnam K et al., Biochemistry 33:6623-30 (1994)).

Alternatively, unprotected peptide segments are chemically linked where the bond formed between the peptide segments as a result of the chemical ligation is an unnatural (non-peptide) bond (Schnolzer, M et al. Science, 256:221 (1992)). This technique has been used to synthesize analogs of protein domains as well as large amounts of relatively pure proteins with full biological activity (deLisle Milton RC et al., Techniques in Protein Chemistry IV. Academic Press, New York, pp. 257-267 (1992)).

3. Process for Making the Compositions

Disclosed are processes for making the compositions as well as making the intermediates leading to the compositions. For example, disclosed is the peptide for SEQ ID NOs: 7, 9, 11, 13, and 15. There are a variety of methods that can be used for making these compositions, such as synthetic chemical methods and standard molecular biology methods. It is understood that the methods of making these and the other disclosed compositions are specifically disclosed.

The HBU can be used in a vector for plasmid construction. Basic recombinant DNA methods like plasmid preparation, restriction enzyme digestion, polymerase chain reaction, ligation, transformation and protein synthesis were performed according to well-established

protocols familiar to those skilled in the art,[61] or as recommended by the manufacturer of the enzymes or kit.

Disclosed is a method for making a fusion protein construct comprising amplifying a reporter nucleic acid, restricting the amplified reporter nucleic acid, and ligating the restricted reporter nucleic acid to an HBM nucleic acid, thereby creating a fusion protein construct. Optionally, an additional step of transforming a bacterial host with the fusion protein construct can then be carried out. The HBM nucleic acid can be fused to a GST nucleic acid prior to ligating the HBM nucleic acid to the restricted reporter nucleic acid.

Also disclosed is a method for making a fusion protein nucleic acid, comprising amplifying a reporter nucleic acid, restricting the amplified reporter nucleic acid, and ligating the restricted reporter nucleic acid to an HBM nucleic acid, thereby creating a fusion protein nucleic acid. Optionally, an additional step of transforming a bacterial host with the fusion protein nucleic acid can then be carried out. The HBM nucleic acid can be fused to a GST nucleic acid prior to ligating the HBM nucleic acid to the restricted reporter nucleic acid. The fusion protein can then be expressed and purified.

One method of making an HBM construct comprises amplifying RHAMM cDNA, for example (SEQ ID NO: 7), digesting the amplified RHAMM, ligating the amplified RHAMM into a vector, and obtaining a product from the vector. The method can further comprise introducing a linker into the product, linearizing the vector, and ligating the product into the vector then obtaining a second product from the vector. These steps can be repeated to obtain a third product from the vector as well.

In one example, a 62-amino acid heparin binding domain with two base-rich BX_7B motifs can be used as an individual HBU, and the units can be linked together to form an HBM (this is the HABD molecule referred to above). For example, RHAMM(518-580) cDNA (the 62-amino acid heparin binding domain) can be inserted in a vector such as pGEX-ERL. Primers with cleavage sites can then be used to amplify RHAMM(518-580), and the PCR product can then be digested with and ligated into the modified pGEX vector that had been also digested to obtain a construct. This construct is referred to as HB1. A linker, such as (GlySer)9Gly can then be introduced into the vector and then ligated with another cDNA that had been digested to give an HB2 recombinant construct. This construct is considered a heparin binding molecule (HBM). Furthermore, an HB3 construct can be synthesized by repeating the steps above with another linker and amplified cDNA. This

construct is also considered an HBM. Each of the plasmids, as well as the empty vector, can then be transformed into a bacterial host. The desired peptide can then be purified.

Fusion proteins can be created in order to facilitate detection or purification. One method of making a fusion protein nucleic acid comprises ligating an HBM nucleic acid into a reporter plasmid, thereby creating a fusion protein nucleic acid. The fusion protein can then be expressed and purified. For example, a fusion protein can be made using the GST molecule, as disclosed above. Examples of creating a GST fusion molecule are well described in the art and one of ordinary skill would be able to create such a fusion protein[62].

Fusion proteins can also be created in order to express chromogenic and fluorescent dyes. Various fluorescent and chromogenic dyes are disclosed above. The fusion protein can be created by using a plasmid inserted into a host. The host can be any cell capable of producing a fusion protein. One of ordinary skill in the art would be able to use a host to form such a fusion protein. The host can be bacterial, such as *E. coli*, for example. In one example, to create fusion proteins, *E. coli* expression plasmids can be generated that carry fusions of the appropriate gene fragments. They can be generated by PCR amplification of the EGFP gene, for example, or the BAP gene, using tailed primers with restriction sites. Following the appropriate restriction digestions, these fragments can be ligated into the HBM gene to create terminal fusions. Following transformation, protein products can be expressed and purified using standard purification techniques.

EGFP, BAP, and GST-HBM are readily expressed in soluble form in *E. coli*, for example. Once expressed, all three proteins are relatively stable in a variety of salt, detergent, pH, mildly oxidizing, and denaturing buffers. This allows flexibility to modify purification or assay methods. The HBM gene can also be placed in EGFP and pFLAG-BAP, for example, utilizing restriction sites. pFLAG-BAP carries an OMP-A leader peptide, which results in the secretion of the fusion protein into culture media. Growth of *E. coli* in defined media will allow direct purification by ion-exchange chromatography. Isolation of EGFP-HBM can be achieved using an anti-GFP affinity column.

C. Methods of Using

Heparin is a highly heterogeneous glycosaminoglycan (GAG), a family of polysaccharides with alternating uronic acid and aminoglycoside residues that is extracted from mast cells of porcine intestinal mucosa or bovine lung. The chemical modifications, particularly sulfation, lead to pentasaccharide sequences that serve as binding sites for antithrombin III (AT III).[2] In blood, heparin interacts with AT-III, which blocks activation

of factor Xa and thereby prevents blood coagulation.[3] The anticoagulant effect of heparin is mediated through this interaction, which markedly accelerates the rate of AT III inhibition of thrombin (factor IIa) and factor Xa.

Two kinds of heparin, unfractionated free heparin (UFH) and low molecular weight heparin (LMWH), are employed as therapeutic agents to reduce blood clot formation and thrombosis.[4-8] Unfractionated heparin (UFH) polysaccharides are heterogeneous in length and anticoagulation activity and range in mass from 5000 to 30,000 Da. Low-molecular-weight heparins (LMWH) are produced from unfractionated heparin to yield smaller polysaccharides with average molecular masses of 4000–5000 kDa. These shorter molecules lose the ability to accelerate AT III inhibition of thrombin but retain the ability to catalyze factor Xa inhibition. Decreased *in vivo* protein binding improves LMWH bioavailability and leads to more predictable anticoagulant response. Another important aspect of LMWH treatment is that it may be administered as a subcutaneous injection as opposed to an intravenous administration of UFH.

Plasma heparin levels can be detected by several clinically-approved methods: (i) determination of activated coagulation time (ACT), (ii) activated partial thromboplastin time (APTT)[12], (iii) the heparin management test (HMT) [13,14] or (iv) the anti-factor Xa assay.[15] Another chemical method measured heparin by monitoring inhibition of thrombin activity on a fluorogenic substrate[16]; however, this method lacked the sensitivity required for clinical use. For over 30 years, the measurement of APTT has remained the most widely used tool for prescribing and monitoring the use of anticoagulants in patients.

The APTT is a global screening test of coagulation used to evaluate the intrinsic coagulation pathway. It is affected by many variables in addition to heparin, including coagulopathies, inhibitors, and increases of factor VIII and fibrinogen. Secondly, there is no agreement on what value should be used for the denominator of APTT ratios: mean or upper limit APTT of a reference range for normal, or a patient's pretreatment APTT. Most importantly, commercial APTT reagent sensitivities to heparin vary widely. In addition, there are potential surface-to-volume effects when small samples are employed, and the effects that sample processing can have on both the coagulation and thrombotic pathways. Collectively, these factors can introduce significant analytical error when performing an APTT.[1,17]

The anti-factor Xa assay is a chromogenic assay that is based on heparin's ability to inactivate factor Xa in the clotting cascade. In this method, both factor Xa and antithrombin

III are present in excess and the residual factor Xa activity is inversely proportional to the heparin concentration. The assumption is made that the patient has a normal concentration of antithrombin III. It is recommended to also measure the antithrombin III levels for all patients when using the anti factor Xa assay. During LMWH therapy there are highly significant differences between anti factor Xa activity results obtained with different assays. The mean of results by one technique have been more than twice those by another. This poor level of agreement between results obtained with some anti factor Xa assays suggests that the management of patients may be hampered by the laboratory technique that is performed to monitor them. The largest difference between results with different chromogenic techniques was 43%. The reason for differences between results with one clotting assay and other clotting or chromogenic assays is unknown but may relate to the influence of thrombin inhibition during the assay. The composition of LMWH changes after administration with the rapid loss of anti IIa activity. Some clotting based assays are probably influenced by the anti IIa activity, which remains in the heparin, added to plasma to construct the calibration curve. This material is largely missing from the test sample, which is collected from patients 4-6 hours after injection. Thus the clotting times used to establish the calibration curve are prolonged in relation to the test sample, leading to a systematic underestimation of the anti-Xa activity. Only assays uninfluenced by anti IIa activity would not show this effect.[18] These disparate readouts underline the importance of having an assay that measures heparin directly, rather than assessing a physiological indicator of the clotting cascade.

Protamine sulfate is naturally-occurring cationic protein that is routinely used to neutralize heparin in a wide variety of clinical procedures, including cardiovascular surgery, hemodialysis, and cardiac catheterization.[23,24] Removal or neutralization of heparin restores the patient's native coagulation state. However, adverse reactions – e.g., anaphylactic shock, systemic hypotension, thrombocytopenia, granulocytopenia, complement activation, and cytokine release- can result from protamine use.[25] Alternative methods currently include extracorporeal affinity-based heparin adsorption by a so-called heparin removal device (HRD), or use of heparinase to degrade the heparin.[26] Such devices may use immobilized poly-L-lysine (PLL)[27], protamine-immobilized cellulose filters[23,24], or other polycationic ligands.[28,29] Using PLL , the HRD requires 0.5-2 hr for 90% reduction of heparin in blood, and employs an exchange cell in which the heparin diffuses out of the plasma and is trapped on the bead-immobilized affinity ligand. A combination approach, i.e., adding a

polyethylene glycol (PEG) 3400 linker, and using 100-kDa PLL pre-coating of the fiber membranes, substantially amplifies the protamine removal properties. A small cartridge can adsorb 60 mg/g fiber, an 8-fold enhancement over immobilized protamine alone. Immobilized heparinase has also been evaluated for extracorporeal heparin removal.[30] Nonetheless, capacity and selectivity are problems inherent to all current methods in use.

Alternatively, heparin can be neutralized by binding to an HBM. For example, heparin that doesn't bind to the protamine sulfate, such as synthetic heparins, can be bound by an HBM. Protamine sulfates and HBMs can be used in conjunction, or the HBM can be used in place of protamine for neutralization of the anticoagulant effects of heparin. HBM can be used in smaller quantity, thereby alleviating the negative effects of protamine. HBM can bind to heparin molecules that protamine does not bind, including synthetic heparins. HBM does not have the allergic effects that protamine can have in some subjects.

During surgical procedures when a patient's blood contacts uncoated medical devices, the device surfaces modify plasmatic proteins, promote platelet aggregation, and activate the complement system, unleashing thrombus formation. Thus, it becomes necessary to use an anticoagulant to keep these events from starting. Heparin is the anticoagulant most used for this purpose and is typically immobilized onto the surface of these medical devices. Heparin immobilization can be accomplished by microwave-plasma activation of polypropylene fabrics, followed by grafting of acrylic acid and covalent heparin binding through amide linkages.[31] Alternatively, a non-cytotoxic crosslinked collagen suitable for endothelial cell seeding was modified with N-hydroxysuccinimide and carbodiimide chemistry, coupling collagen lysine residues to heparin carboxylates.[32] Another alternative is to modify hydrophobic device surfaces by ionic complexation using a polymerizable cationic lipid to form a 60 nm thin layer.[33] All surfaces are subject to patchiness or modification and crazing/cracking as a result of flexing of the surface. Determining the uniformity of heparin coating is an important area of quality control (QC).

QC to show the success of heparin immobilization on devices often consists of testing for adsorbed proteins and soluble activation markers such as antithrombin, thrombin, high-molecular-weight-kininogen (HMWK), and fibrinogen binding capacity.[34,35] Others have used clinical methods such as APTT or anti-factor Xa methods to determine the anticoagulant activity of a heparin coating[36] or the relative surface content of sulfur to demonstrate immobilization of heparin on a blood pump.[37] Platelet activation and flow cytometry in a whole blood assay has been employed to test heparin-coated tantalum stents

and gold-coated stainless steel stents.[38] Similarly, anti-thrombogenicity using APTT, platelet adhesion, and thrombin generation were evaluated in heparin, fibronectin, and recombinant hirudin-coated Nitinol coils designed for closure of intra-atrial communications.[39] Importantly, none of the currently used methods directly detects heparin coatings. The present methods of heparin detection improves and simplifies quality control of these medical devices, and is useful for validating the homogeneity of heparin coating on the devices.

1. Methods of Detecting Heparin

The disclosed compositions can be used as a method of detecting heparin. It is understood that the methods can be used to detect or bind any heparin molecule, including those recited herein. Furthemore the HBMs and HBUs and HABDs can be used to detect to bind any heparin molecule, including those recited herein. Various assays can be used in to detect heparin, including ELISAs, fluorescent based assays, APTT (Activated Partial Thrombin Time) assays, and others disclosed herein. Furthermore, assays can be used in order to quantify the amount of heparin in a sample. One example of a method for determining the amount of heparin in a sample comprises incubating the sample with an HBM in a first incubation, thereby forming a HBM mixture, wherein the HBM mixture allows for the formation of an HBM-heparin complex

Heparin can be detected in blood, plasma, serum, urine, sputum, peritoneal fluid, or any other bodily fluid for which analytical data are desired. Heparin can also be visualized on a coated surface.

Both low molecular weight heparins (LMWH) and unfractionated heparin (UFH) can be detected by the methods described herein. The HBMs disclosed herein bind all major unfractionated heparins, such as bovine, porcine, Sigma, high antithrombin affinity fraction, high affinity fraction, and inactive portions of heparin (figures 23 and 25). High affinity heparin fractions are able to bind to AT III, while inactive fragments are defined as those fragments not capable of binding to AT III. The detection assays described herein can detect inactive fragments. Inactive fragments can be quantitated, for example, by using an assay that detects only heparin that binds AT III in conjunction with the assays described herein that bind either inactive or active portions of heparin, and conducting a a subtraction assay to determine the amount of bound inactive heparin. These inactive fragments are useful to control inflammation, for example, and can be used in a manner similar to chondroitin sulfate. Unfractionated heparin can also be detected over an extended range, for example,

Figure 27 shows the detection of heparin at less than 0.1 U/ml concentrations.

LMWHs include those found in Table 4, for example, all of which are detectable by the methods described herein.For example, in Figures 22 and 24, dalteparin, enoxaparin, tinzaparin, ardeparin, and parnaparin are all able to be bound by the HBMs described herein. Synthetic heparin can also be readily detected (Figure 26).

Table 4: Low Molecular Weight Heparins

Name	Manufacturer	Trade Names	Defractionation Method	Average Molecular Weight (daltons)	Anti Xa:IIa Ratio
Ardeparin sodium	Wyeth-Ayerst	Normiflo RD 11885 WY-90493-RD	Peroxidative depolymerization	5500-6500	1.8:1
Certoparin sodium	Novartis	Alphaparin Mono-Embolex NM Sandoparin Troparin	Amyl nitrate degradation	6000	2:1
Dalteparin sodium	Pharmacia	Fragmin Boxol FR 860 Kabi 2165 Low Liquemine Tedelparin	Nitrous acid depolymerization	5600-6400	2:1
Enoxaparin sodium	Aventis	Lovenox Clexane Decipar Enoxaparine Pharmuka 10169 PK-10169 Plaucina RP-54563 Thrombenox	Benzylation and alkaline depolymerization	4500	2.7:1
Nadroparin calcium	Sanofi-Winthrop	CY-216 Fraxiparin Fraxiparina Fraxiparine Seleparina	Nitrous acid depolymerization	4300	3.2:1
Parnaparin sodium	Aventis	Alpha LMWH Fluxum Minidalton	Cupric acid and hydrogen peroxide degradation OP-21-23	4500-5000	3:1
Reviparin sodium	Knoll	Clivarin LU 473111	Nitrous acid degradation	4150	3.5:1
Tinzaparin sodium	DuPont Pharma	Innohep Logiparin	Enzymatic degradation	6500	1.9:1

		Novo LHN 1			

It is understood that these and other LMWH and heparin molecules are and can be used for administration to a subject in need of anticoagulation properties for a period of time. As these molecules are metabolized at different rates and different amounts, for example, can be given, it is advantageous to be able to monitor the amount of the administered heparin or LMWH, for example, in real time. It is understood that the disclosed HBMs and HABD and methods of using them are capable of performing this monitoring.

Also disclosed are methods of restoring blood coagulation paramaters in a subject in need thereof.

a) Method of Detecting Heparin in a Sample

One method of detecting heparin comprises obtaining a sample, applying the sample to an assay, wherein the assay utilizes an HBM, and detecting the heparin. Also contemplated is a method comprising obtaining a sample, contacting the sample with an HBM, and assaying for HBM-heparin complexes. Also contemplated is a method comprising mixing an HBM and heparin sample together, forming an HBM mixture, and determining if an HBM-heparin complex is present. Specific embodiments are disclosed below. As described above, all types of heparin molecules can be detected, both long and short chain, as well as synthetic heparin.

Any type of synthetic heparin can be detected. Examples include idraparinux (a pentasaccharide, available from Sanofi-Synthelabo) and sulphaminoheparosansulphates such as those found in US Patent 6,329, 351, herein incorporated by reference in its entirety for its teaching regarding synthetic heparins.

Heparin detection assays can be used in quality control, pharmacokinetics, protamine sulfate optimization, and correlation assays to determine heparin antiproliferative and anti-inflammatory effects. Heparin detection assays can also be used to measure oral, inhalation, and depo-administered heparin in a subject, and to measure leaching of heparin speharose or heparin-coated medical devices such as stents.

(1) ELISAs

ELISAs are widely used in clinical research and diagnostics. Any standard ELISA plate can be used with the disclosed embodiments, including but not limited to 96 and 384

well formats. Both the traditional unfractionated heparin (UFH) as well as low molecular weight heparins (LMWH) can be used.

(a) Competitive ELISAs

Due to the hydrophilic nature of heparin, streptavidin-coated microtiter plates treated with commercially available biotinylated heparin can be used. After a wash step, the wells are blocked and stabilized with a protein free coating solution. The HBM reagent is then added to the analyte (which can come from a known or an unknown sample) for which heparin levels are being determined and allowed to equilibrate. The HBM-analyte mixture is then added to the wells of the heparin coated microtiter plate. The coating method can be using biological linkers such as streptavadin (on a plate, for example) and biotin (on heparin, for example) or any number of other linkers such as antibody/antigen pair, GST/GSH, and others which are known in the art. Heparin can also be directly conjugated to plastic using NHS-heparin (N-hydroxysuccinimide heparin) or other activators. For example, 50 to 50,000 ng/ml biotinylated heparin on streptavidin plates can be used. 100 to 10,000 ng/ml can also be used. More heparin on the plate gives the ability to detect high ranges of heparin in samples, while low levels of heparin on the plate gives a more sensitive test, allowing assay of lower levels of heparin. The heparin from the sample and the immobilized heparin then compete for heparin binding sites on the HBM. Binding of the HBM to the immobilized heparin can be detected using a secondary reagent such as HRP conjugated antibody that recognizes the HBM via a tag, such as GST. This is followed by detection of secondary reagent activity using a detection agent such as TMB. Color development can then be stopped and absorbance can be measured. The signal produced is inversely proportional to the amount of heparin present in the analyte, as the heparin of the analyte competes for the HBM binding to the heparin coated plate. A series of increasing concentrations of heparin can be performed in conjunction with the assay to allow for determination of the amount of heparin present by comparison to the standard curve. In one embodiment, the capture protein is GST-HB3 fusion protein in which the GST has been cleaved, and the remaining HB3 protein is utilized as the capture protein.

Fluorescent-based methods can also be used to visualize HBMs bound to heparin. For example, the HBM can be fused with a fluorescent molecule such as BAP or GFP, for example. Alkaline phosphatase fusion constructs are routinely used in subcellular protein localization. In addition to fusion constructs, fluorescent dyes can be chemically conjugated to the HBM.

Plasma, serum, or blood can be used as the analyte. A serum based heparin assay eliminates the need for drawing a separate citrated tube of blood, thus decreasing the total volume of blood needed to be drawn from a patient. A serum based heparin assay allows the sample to come from the same tube of blood as for other assays. In subjects having only a heparin level drawn, there is a need to draw an additional tube of blood prior to drawing a citrated tube, as a means of clearing the activated tissue factor proteins that would affect a clotting cascade based assay. The elimination of this extra tube provides both time and cost savings. The assay can be optimized using different amounts of HBM or other reagents. A multivariate experimental design program can be used to optimize the results. One example of a multivariate experimental design is the ECHIP program. Variables can include pH, constitution of buffers, timing for incubations, and concentrations of biotinylated heparin, HBM, and conjugated antibody. The heparin can be UFH or LMWH.

(b) Sandwich format ELISAs

In a sandwich assay format, the detection signal increases with increasing heparin concentrations in the analyte rather than decreasing, as is the case with the competitive assay format described above. First a "capture protein" is selected to coat the wells. In one example, HB3-GST is used as the HBM molecule, however, any of the coating methods described herein can be used in the sandwich format ELISA. The GST tag of the HB3 protein is cleaved and then the cleaved HB3 is immobilized in the wells of a microtiter plate as the capture molecule.

An alternative approach is to utilize a completely different polycationic species as the capture ligand. This has the advantages of avoiding aggregation, being more economical and easy to prepare in advance, and provide two different affinity ligands for maximal differentiation. First, capture ligands are employed. Examples of such capture ligands include protamine and poly-L-lysine (PLL). Synthetic polycationic polymers can also be used. The polycationic polypeptide is adsorbed and coated to the wells. Following a wash step, the analyte is then be added to the wells and allowed to equilibrate. After washing off unbound analyte, HBM is added to the wells. Binding of the HBM to the heparin can be detected using the HRP conjugated anti-GST antibody as in the competitive assay, for example (Example 8). This step can be followed by colorimetric detection of the HRP activity with TMB. Color development is stopped by acidification, and absorbance read. Signal increases as increased amounts of heparin in the analyte are captured by the capture protein. A series of heparin standards can be used as controls in this assay format.

Importantly, the sandwich format provides increased signal with increasing heparin in the sample being analyzed. In contrast to APTT or anti-Xa assays, direct heparin detection can be performed in serum, rather than plasma, as it does not rely on the clotting cascade. As with the competitive assay, a multivariate experimental design can be used to optimize this assay. The assay can be performed in blood, plasma, or serum, for example.

(2) Fluorescent Based Assays

A fluorescent-based assay can be used for both UFH and LMWH. By way of example, streptavidin-coated microtiter plates can be used which have been treated with biotinylated heparin. After a wash step, the wells can be blocked and stabilized with a protein free coating solution. If, by way of example, BAP is used as the fluorescent molecule, the BAP-HBM reagent can be added to the analyte for which heparin levels are being determined and allowed to equilibrate. This BAP-HBM-analyte mixture is then added to the wells of the heparin coated microtiter plate. The unknown heparin and the immobilized heparin will compete for heparin binding sites on the BAP-HBM. Binding of the BAP-HBM to the plate can then be detected colorimetrically using a substrate that will react with the BAP tag present on the HBM. Color development is stopped and the absorbance is measured. The signal produced will be inversely proportional to the amount of BAP-HBM binding to the heparin coated plate.

(3) Quantification

The level of heparin can be quantified utilizing an HBM. For example, the amount of heparin in plasma can be determined by spiking the plasma with heparin calibration standards. Figure 24 shows the measurement of enoxaparin in plasma plotted in log. Figure 25 shows that unfractionated heparin can also be measured quantitatively in plasma. Competitive and sandwich assay formats can be compared with identical samples. Aliquots of plasma can be mixed with equal volumes of serial dilutions prepared from heparin. Relative absorbance vs. heparin concentration (log/log) can then be plotted to obtain calibration curves. By way of example, the optimal range for heparin measurements is from 100 ng/ml to 2000 ng/ml for UFH and from 400 ng/ml to 2000 ng/ml for LMWH. With parallel Anti-Xa assay experiment, this corresponds to 0.1-5 U/ml for UFH and 0.3-2 U/ml for LMWH, suitable for therapeutic levels in plasma, which are generally between 0.1-1.0 U/ml. In one example of a quantitation assay, heparin was bound to the inside of a a microplate well (Figure 18). Various concentrations of standards were then placed in the wells, which amounts can vary according to the need thereof (Figure 19). Unknown heparin

concentrations were then placed in the remaining wells, and a competitive binding reaction using HRP-HB3 was carried out. The sample was then incubated, and a reaction took place between the HB3 and the heparin (Figure 20). In low heparin samples, more HBM-HRP is available to bind, creating a stronger signal. In high heparin samples, less HBM-HRP is available, creating a weaker signal.

The HBM is capable of detecting levels of heparin between 1ng/ml to 100,000 ng/ml. The HBM is capable of detecting levels of heparin between 10 ng/ml and 10,000 ng/ml. The HBM is capable of detecting levels of heparin between 100 ng/ml to 2000 ng/ml.

b) Method of Detecting Heparin on a Coated Surface

During surgical procedures when a patient's blood contacts uncoated medical devices, the device surfaces modify plasmatic proteins, promote platelet aggregation, and activate the complement system, unleashing thrombus formation. Thus, it is necessary to use an anticoagulant to keep this process from starting. Heparin is an anticoagulant most used for this purpose and is typically immobilized on to the surface of many surgical instruments and instruments for use in hospitals. Because of the tremendous importance of these instruments having an appropriate, evenly-applied layer of heparin, quality control of these instruments is vital. Furthermore, heparin application to instruments in solution tends to degrade over time, due to cations in solution that attach to the anions on the chain, removing the bond to the cation on the surface and allowing that part of the chain to enter the solution.

Also important are heparin coated stents, which are used to combat the issue of restenosis following angioplasty. Quality control of these stents using the methods disclosed below allows for the visualization of the uniformity of heparin coating on a stent, saving time and money compared to the standard quality control methods now employed.

One method of detecting heparin on a coated surface comprises conjugating the HBM to HRP, then detecting the HRP by fluorescence, colorimetry, or chemiluminescence. Another method of detecting heparin on a coated surface comprises exposing the surfaces to an HBM fused to a reporter molecule, washing the coated surface to remove excess HBM fused to the reporter molecule, and assaying for the reporter molecule. In one embodiment, the reporter molecule can be visualized and the uniformity of heparin on the coated surface determined.

As mentioned above, HBMs fused to fluorescent reporter molecules can be used, by way of example. The device surface is exposed to the HBM fusion protein, and then fluorescent microscopy can be utilized to detect the level of fluorescence given off by the

surface. Flexing and recollapsing of the instrument or stent cracks and grazes the coating so discontinuities can be visualized. Fluorescence can be detected by, for example, using microscopy, or other detectors.

c) Medical Devices Coated with HBMs

Another embodiment described herein is an apparatus comprising an implantable medical device, such as a stent, which can be coated with HBMs during manufacture. These devices have an advantage over other medical devices as they can alleviate the need to coat these devices directly with heparin. In aonther embodiment, heparin can be secondarily coated onto the device by presenting it to the HBM during manufacture. Alternatively, the device can be implanted into a subject and the attached HBM allowed to bind to endogenous glycosaminoglycans, including heparin.

2. Methods of Removing or Neutralizing Heparin

Neutralizing heparin can be done *in vivo* in order to stop the effects of heparin in a subject. Removing heparin can be done *ex vivo* in order to clear it from the subject. Removing or neutralizing heparin from blood, plasma, or serum is often needed in a clinical setting. Heparin must be removed or neutralized from the blood for surgical or other reasons. For example, when patients undergo cardiac surgical procedures, such as angioplasty or coronary artery bypass graft surgery, blood thinners such as heparin are commonly administered prior to the procedure to prevent blood clots. Blood tends to clot when subjected to foreign instruments, such as a bypass machine or balloons used in angioplasty. The heparin can be removed by immobilizing an HBM, exposing the HBM to a sample, and removing the heparin from the sample of fluid. Affinity chromatography can be used, for example, to remove heparin from a sample. HBMs can also be used to neutralize heparin by administering it to a subject in need thereof.

Heparin can be removed from the sample at the rate of 1 to 10%, 10 to 20%, 20 to 30%, 30 to 40%, 40 to 50%, 50 to 60%, 60 to 70%, 70 to 80%, 80 to 90%, and 90 to 100% of total heparin removed.

The removal of heparin can take from 1 minute to 48 hours, from 1 hour to 24 hours, or from 4 hours to 12 hours.

The following are examples of specific methods that can be used to remove heparin.

a) Adsorbing to Beads

One method of removing heparin from involves adsorbing the heparin to beads. In one example, a GST-HBM construct is adsorbed to glutathione-Sepharose, in the identical manner employed for purification of GST-HBM. This anchors the HBM by the high affinity, but non-covalent, GSH-GST interaction. The HBM can also be biotinylated for use with companion streptavidin beads. The sample containing heparin to be removed is then contacted with the beads, thereby causing an HBM-heparin interaction which removes the heparin from the sample. HBM an also be activated and applied to a bead. In another embodiment, HBM can be applied to activated beads.

HBM binds more strongly to longer chained heparins allowing an affinity purification of heparin based on molecular size. Varying the number of HBU in the HBM used for purification can allow preferential binding of various sizes of heparin molecules.

b) Covalently Attaching

In another method, the HBM is covalently attached to beads. In one example, a GST-HBM construct is covalently attached to AffiGel-10 NHS-activated beads by formation of an amide linkage between lysine residues of GST and the activated ester of the agarose beads. This has the possibility of modifying an HBM lysine residue, but a significant number of linkages will still occur to GST, and only those linkages that preserve HBM-heparin binding are important.

c) Reductive Amination

An HBM can also be linked by reductive amination to a bead. By way of example, a GST-HBM can be linked by reductive amination with $NaBH_3CN$ at pH ranging from 4.0 to 6.0, more specifically in the range of pH 4.5 to 5.5, more specifically at pH 5.0, to a periodate-activated-epoxy-activated agarose bead. The resulting secondary amine linkage to protein lysine residues also covalently immobilizes the heparin-binding domain. The beads are then exposed to a heparin-containing sample, and the heparin is immobilized on the beads.

D. Kits

Disclosed herein are kits that are drawn to reagents that can be used in practicing the methods disclosed herein. The kits can include any reagent or combination of reagent discussed herein or that would be understood to be required or beneficial in the practice of the disclosed methods. For example, the kits could include primers to perform the

amplification reactions discussed in certain embodiments of the methods, as well as the buffers and enzymes required to use the primers as intended.

An example of a kit for a heparin ELISA comprises a microplate, an HBM, and a color-developing reagent, control standards, a wash buffer, and instructions such as the Accucolor Heparin Kit from Sigma, control standards, such as, heparin salt products, and wash buffers such as, PBS or TBS with detergent Tween-20 added. The microplate can be, for example, a heparin coated or HBM-coated microplate. The HBM can optionally be linked to an enzyme for detection. Instead of an HBM-enzyme, the kit can optionally include an HBM-GST and anti-GST-HRP. Examples of kits and instructions for their use can be found in Examples 9-12, for example.

Another example of a kit comprises a bedside heparin quick test. This kit comprises an immunochemical test, and instructions. The immunological test can be similar to a one step pregnancy test. For example, the test can comprise a strip that containing an HBM and a molecule that changes color when heparin is detected. For example, a sample of urine or blood can be placed in an application window. The fluid fraction along with its dissolved components including the heparin, move along with the liquid front. When the fluid reaches the HBM, which can be in great excess, the heparin can react with the HBM. When this happens, the HBM triggers an enzyme to start making an insoluble dye, which upon accumulating causes the vertical bar on the "plus sign" to become visible. The test can optionally include a control window. The control window shows a plus to indicate that the HBM in the paper had not become damaged. The test can use urine, blood, sputum, serum, or plasma, for example, to detect heparin.

Another example of a kit includes an HBM fused to a fluorescent molecule. The HBM can be a fusion protein, for example. The fluorescent molecule can be any fluorescent molecule capable of allowing for the detection of the HBM. One of skill in the art will readily understand which fluorescent molecules can be used. Examples include GFP and BAP. This kit can also comprise any of the various HBM molecules and their variants disclosed above.

Another example of a kit includes an extracorporeal heparin removal device (HRD) kit. This kit comprises an HBM molecule as an affinity capture ligand Basically, in one example, sterilized beads containing immobilized HBM would be contained in a sterile tube through which a bodily fluid such as blood would be passed. The heparin would be captured on the beads while the remaining fluid constituents would pass through un-

retained. The captured heparin could be released later by elution with a low pH and or high-salt buffer for analysis, if desired.

E. Sequences

1. SEQ ID NO: 1 BX7B (B is either R or K and X7 contains no acidic residues and at least one basic amino acid)

2. SEQ ID NO: 2

5'-CGGGATCCGGTGCTAGCCGTGACTCCTATGCACAGCTCCTTGG-3'

3. SEQ ID NO: 3

5'-GGAGCGGTCGACACGGATGCCCAGAGCTTTATCTAATTC-3'

4. SEQ ID NO: 4

5'-GATCCGGTCTCGAGGGAAGTGGTTCTGGAAGTGGTTCAGGTTCGGGTAGCGGA
TCTGGTTCAGGAAGTGGTT-3'

5. SEQ ID NO: 5

5'-CTAGAACCACTTCCTGAACCAGATCCGCTACCCGAACCTGAACCACTTCCAGA
ACCACTTCCCTCGAGACCG-3'

6. SEQ ID NO: 6

RDSYAQLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRSQLVKRKQNELRLQGELD
KALGIR

7. SEQ ID NO: 7 hyaluronan mediated motility receptor (RHAMM) [Mus musculus].

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ACCESSION   NP_038580
VERSION     NP_038580.1  GI:7305145
DBSOURCE    REFSEQ: accession NM_013552.1

      1 msfpkaplkr fndpsgcaps pgaydvktse atkgpvsfqk sqrfknqres qqnlsidkdt
     61 tllasakkak ksvskkdsqk ndkdvkrlek eirallqerg tqdkriqdme selekteakl
    121 naavrektsl sasnaslekr lteltranel lkakfsedgh qknmralsle lmklrnkret
    181 kmrsmmvkqe gmelklqatq kdlteskgki vqlegklvsi ekekidekce teklleyiqe
    241 iscasdqvek ckvdiaqlee dlkekdreil slkqsleeni tfskqiedlt vkcqlleter
    301 dnlvskdrer aetlsaemqi lterlalerq eyeklqqkel qsqsllqqek elsarlqqql
    361 csfqeemtse knvfkeelkl alaeldavqq keeqserlvk qleeerksta eqltrldnll
    421 rekevelekh iaahaqaili aqekyndtaq slrdvtaqle svqekyndta qslrdvtaql
    481 eseqekyndt aqslrdvtaq leseqekynd taqslrdvta qlesvqekyn dtaqslrdvs
    541 aqlesyksst lkeiedlkle nltlqekvam aeksvedvqq qiltaestnq eyarmvqdlq
    601 nrstlkeeei keitssflek itdlknqlrq qdedfrkqle ekgkrtaeke nvmteltmei
    661 nkwrllyeel yektkpfqqq ldafeaekqa llnehgatqe qlnkirdsya qllghqnlkq
    721 kikhvvklkd ensqlksevs klrsqlvkrk qnelrlqgel dkalgirhfd pskafchask
    781 enftplkegn pncc
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8. SEQ ID NO: 8 hyaluronan mediated motility receptor (RHAMM) [Mus musculus] nucleic acid.

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ACCESSION   NP_038580
VERSION     NP_038580.1  GI:7305145
DBSOURCE    REFSEQ: accession NM_013552.1

tcaggcgagc tgacagtttg ctggggcgtt tgattgctgt ctcatctgga cccaggcgtc
      61 agaatgtcct ttcctaaggc gcccctgaag agattcaatg acccttcggg ttgtgctcca
     121 tctccgggtg cttatgatgt taaaacttca gaagcaacta aaggaccagt gtcttttcag
     181 aaatcacaaa gatttaaaaa ccaaagagag tctcaacaaa atcttagcat tgacaaagat
     241 acaaccttgc ttgcttcggc taaaaaagca aagaagtctg tgtcaaagaa ggactctcag
     301 aagaatgata aagatgtgaa gagattagaa aaagagattc gcgctctttt gcaagagcga
     361 gggactcagg acaaacggat ccaggacatg gaatctgaat tggagaagac agaagcaaag
     421 ctcaatgcag cagtcagaga gaaaacatct ctctctgcga gtaatgcttc actggaaaaa
     481 cggcttactg aattaaccag agccaacgag ctactaaagg ctaagttttc tgaagatggt
     541 caccaaaaga atatgagagc tctaagcctg gaattgatga aactcagaaa taagagagag
     601 acaaagatga ggagtatgat ggtcaaacag gaaggcatgg agctgaagct gcaggccact
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    1261 aaacagctgg aagaggaaag gaagtcaact gcagaacaac tgacgcggct ggacaacctg
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    3241 aaatcaaact acaacctcaa acgtctggga ccctccaagg gcttgggtca cttctctagc
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```

9. SEQ ID NO: 9 (M.musculus mRNA RHAMM).

```
ACCESSION   X64550 S41029
VERSION     X64550.1  GI:1495185
KEYWORDS    cell motility; hyaluronic acid receptor; RHAMM gene.
SOURCE      Mus musculus (house mouse)
```

```
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EGNPNCC"
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10. SEQ ID NO: 10 (M.musculus mRNA RHAMM) nucleic acid

```
ACCESSION   X64550 S41029
VERSION     X64550.1  GI:1495185
KEYWORDS    cell motility; hyaluronic acid receptor; RHAMM gene.
SOURCE      Mus musculus (house mouse)
```

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 481 gagaaggatc gtgagatttt aagtcttaag cagtctcttg aggaaaacat tacattttct
 541 aagcaaatag aagacctgac tgttaaatgc cagctacttg aaacagaaag agacaacctt
 601 gtcagcaagg atagagaaag ggctgaaact ctcagtgctg agatgcagat cctgacagag
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11. SEQ ID NO: 11 Rattus norvegicus Hyaluronan mediated motility receptor (RHAMM)

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ACCESSION    NM_012964
VERSION      NM_012964.1  GI:6981029
SOURCE       Rattus norvegicus (Norway rat)
  ORGANISM   Rattus norvegicus
```

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12. SEQ ID NO: 12 Rattus norvegicus Hyaluronan mediated motility receptor (RHAMM) nucleic acid

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13. SEQ ID NO: 13 Homo sapiens hyaluronan-mediated motility receptor (RHAMM)

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VERSION     NM_012485.1  GI:7108350
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
```

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14. SEQ ID NO: 14 Homo sapiens hyaluronan-mediated motility receptor (RHAMM) nucleic acid

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2761 taagcctgca ttcttaacaa actcttcagt taattcttag atacactaaa aatctgagaa
2821 actctacatg taactatttc ttcagagttt gtcatatact gcttgtcatc tgcatgtcta
2881 ctcagcattt gattaacatt tgtgtaatat gaaataaaat tacacagtaa gtcatttaac
2941 caaaaaaaaa aaaaaaa
```

15. SEQ ID NO: 15 Homo sapiens hyaluronan receptor (RHAMM) mRNA.

```
ACCESSION    U29343
VERSION      U29343.1  GI:2959555
MSFPKAPLKRFNDPSGCAPSPGAYDVKTLEVLKGPVSFQKSQRF

KQQKESKQNLNVDKDTTLPASARKVKSSESKKESQKNDKDLKILEKEIRVLLQERGAQ

DRRIQDLETELEKMEARLNAALREKTSLSANNATLEKQLIELTRTNELLKSKFSENGN

QKNLRILSLELMKLRNKRETKMRGMMAKQEGMEMKLQVTQRSLEESQGKIAQLEGKLV

SIEKEKIDEKSETEKLLEYIEEISCASDQVEKYKLDIAQLEENLKEKNDEILSLKQSL

EDNIVILSKQVEDLNVKCQLLETEKEDHVNRNREHNENLNAEMQNLEQKFILEQREHE

KLQQKELQIDSLLQQEKELSSSLHQKLCSFQEEMVKEKNLFEEELKQTLDELDKLQQK

EEQAERLVKQLEEEAKSRAEELKLLEEKLKGKEAELEKSSAAHTQATLLLQEKYDSMV

QSLEDVTAQFESYKALTASEIEDLKLENSSLQEKAAKAGKNAEDVQHQILATESSNQE

YVRMLLDLQTKSALKETEIKEITVSFLQKITDLQNQLKQQEEDFRKQLEDEEGRKAEK

ENTTAELTEEINKWRLLYEELYNKTKPFQLQLDAFEVEKQALLNEHGAAQEQLNKIRD

SYAKLLGHQNLKQKIKHVVKLKDENSQLKSEVSKLRCQLAKKKQSETKLQEELNKVLG
                                IKHFDPSKAFHHESKENFALKTPLKEGNTNCYRAPMECQESWK"
```

16. SEQ ID NO: 16 Homo sapiens hyaluronan-mediated motility receptor (RHAMM) nucleic acid

```
   1 tcgagcggcc gcccgggcag gtgtgccagt caccttcagt ttctggagct ggccgtcaac
  61 atgtcctttc ctaaggcgcc cttgaaacga ttcaatgacc cttctggttg tgcaccatct
 121 ccaggtgctt atgatgttaa aactttagaa gtattgaaag gaccagtatc ctttcagaaa
 181 tcacaaagat ttaaacaaca aaaagaatct aaacaaaatc ttaatgttga caaagatact
 241 accttgcctg cttcagctag aaaagttaag tcttcggaat caaagaagga atctcaaaag
 301 aatgataaag atttgaagat attagagaaa gagattcgtg ttcttctaca ggaacgtggt
 361 gcccaggaca ggcggatcca ggatctggaa actgagttgg aaaagatgga agcaaggcta
 421 aatgctgcac taagggaaaa aacatctctc tctgcaaata atgctacact ggaaaaacaa
 481 cttattgaat tgaccaggac taatgaacta ctaaaatcta agttttctga aaatggtaac
 541 cagaagaatt tgagaattct aagcttggag ttgatgaaac ttagaaacaa aagagaaaca
 601 aagatgaggg gtatgatggc taagcaagaa ggcatggaga tgaagctgca ggtcacccaa
 661 aggagtctcg aagagtctca agggaaaata gcccaactgg agggaaaact tgtttcaata
 721 gagaaagaaa agattgatga aaaatctgaa acagaaaaac tcttggaata catcgaagaa
 781 attagttgtg cttcagatca agtggaaaaa tacaagctag atattgccca gttagaagaa
 841 aatttgaaag agaagaatga tgaaatttta agccttaagc agtctcttga ggacaatatt
 901 gttatattat ctaaacaagt agaagatcta aatgtgaaat gtcagctgct tgaaacagaa
 961 aaagaagacc atgtcaacag gaatagagaa cacaacgaaa atctaaatgc agagatgcaa
1021 aacttagaac agaagtttat tcttgaacaa cgggaacatg aaaagcttca acaaaaagaa
1081 ttacaaattg attcacttct gcaacaagag aaagaattat cttcgagtct tcatcagaag
1141 ctctgttctt ttcaagagga aatggttaaa gagaagaatc tgtttgagga agaattaaag
1201 caaacactgg atgagcttga taaattacag caaaaggagg aacaagctga aaggctggtc
1261 aagcaattgg aagaggaagc aaaatctaga gctgaagaat taaaactcct agaagaaaag
1321 ctgaaaggga aggaggctga actggagaaa agtagtgctg ctcataccca ggccaccctg
1381 cttttgcagg aaaagtatga cagtatggtg caaagccttg aagatgttac tgctcaattt
1441 gaaagctata aagcgttaac agccagtgag atagaagatc ttaagctgga gaactcatca
1501 ttacaggaaa aagcggccaa ggctgggaaa aatgcagagg atgttcagca tcagattttg
1561 gcaactgaga gctcaaatca agaatatgta aggatgcttc tagatctgca gaccaagtca
1621 gcactaaagg aaacagaaat taaagaaatc acagtttctt ttcttcaaaa aataactgat
1681 ttgcagaacc aactcaagca acaggaggaa gactttagaa aacagctgga agatgaagaa
1741 ggaagaaaag ctgaaaaaga aaatacaaca gcagaattaa ctgaagaaat taacaagtgg
```

```
1801 cgtctcctct atgaagaact atataataaa acaaaacctt ttcagctaca actagatgct
1861 tttgaagtag aaaaacaggc attgttgaat gaacatggtg cagctcagga acagctaaat
1921 aaaataagag attcatatgc taaattattg ggtcatcaga atttgaaaca aaaaatcaag
1981 catgttgtga agttgaaaga tgaaaatagc caactcaaat cggaagtatc aaaactccgc
2041 tgtcagcttg ctaaaaaaaa acaaagtgag acaaaacttc aagaggaatt gaataaagtt
2101 ctaggtatca aacactttga tccttcaaag gcttttcatc atgaaagtaa agaaaatttt
2161 gccctgaaga ccccattaaa agaaggcaat acaaactgtt accgagctcc tatggagtgt
2221 caagaatcat ggaagtaaac atctgagaaa cctgttgaag attatttcat tcgtcttgtt
2281 gttattgatg ttgctgttat tatatttgac atgggtattt tataatgttg tatttaattt
2341 taactgccaa tccttaaata tgtgaaagga acatttttta ccaaagtgtc ttttgacatt
2401 ttattttttc ttgcaaatac ctcctcccta atgctcacct ttatcacctc attctgaacc
2461 ctttcgctgg ctttccagct tagaatgcat ctcatcaact taaaagtcag tatcatatta
2521 ttatcctcct gttctgaaac cttagtttca agagtctaaa ccccagattc ttcagcttga
2581 tcctggaggc ttttctagtc tgagcttctt tagctaggct aaaacacctt ggcttgttat
2641 tgcctctact ttgattcttg ataatgctca cttggtccta cctattatcc tttctacttg
2701 tccagttcaa ataagaaata aggacaagcc taacttcata gtaacctctc tatttt
```

F. References

The following references may be referred to in the specification and each one is specifically herein incorporated by reference.

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G. Examples

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be

accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

1. Example 1: Plasmid Construction

RHAMM(518-580) (SEQ ID NO: 6) cDNA was obtained by PCR from a plasmid containing full length mouse RHAMM. The PCR kit was from Novagen (Madison, WI). The modified vector pGEX-ERL was developed from pGEX by changing endonuclease sites in the multicloning site. A forward primer, 5'-CGGGATCCGGTGCTAGCCGTGACTC CTATGCACAGCTCCTTGG-3' (SEQ ID NO: 2) with BamHI and NheI cleavage sites at 5' and a reverse primer, 5'-GGAGCGGTCGACACGGATGCCCAGAGCTTTATCTAATTC-3' (SEQ ID NO: 3) with a SalI site at 5' were synthesized to amplify RHAMM(518-580). The PCR product was digested with BamHI and SalI and ligated into the modified pGEX vector that had also been digested with BamHI and XhoI to obtain the HB1 construct. This subcloning step eliminates the downstream restriction sites so that the insert cannot be excised during subsequent manipulations. To connect the consecutive multiple copies of the P1 open reading frame (ORF), a $(GlySer)_9Gly$ linker was introduced using the forward primer 5'-GATCCGGTCTCGAGGGAAGTGGTTCTGGAAGTGGTTCAGGTTCGGGTAGCGG ATCTGGTTCAGGAAGTGGTT-3' (SEQ ID NO: 4) containing a XhoI site, and the reverse primer 5'-CTAGAACCACTTCCTGAACCAGATCCGCTACCCGAACCTGAACCACTTCCAG AACCACTTCCCTCGAGACCG-3' (SEQ ID NO: 5) containing a BamHI site. The vector with single P1 ORF was linearized with BamHI and NheI and ligated with the annealed linker primers. This intermediate product was again digested with BamHI and XhoI and then ligated with another PCR-amplified P1 ORF cDNA that had been digested with BamHI and SalI to give the HB2 recombinant construct. The HB3 construct was synthesized by repeating the steps above with another linker and amplified P1 cDNA. All recombinant constructs were sequenced to confirm the presence of in-frame fusions with GST and the absence of mutations that may have been introduced during PCR amplification of RHAMM cDNA.

To obtain a high affinity HA-binding protein, tandem repeats of the region of the RHAMM(518-580) cDNA (Figure 2A) separated by a linker that encoded alternating glycine and serine residues were used. The subcloning scheme is summarized in Figure 2B and was accomplished in five steps: (i) preparation of an engineered GST expression vector

with appropriate restriction sites; **(ii)** insertion of RHAMM(518-580) "P1" domain to obtain the GST-HB1 construct, **(iii)** insertion of an oligonucleotide encoding a 19-residue Gly-Ser linker region (GSGSGSGSGSGSGSGSGSG) to separate P1 domains, **(iv)** addition of a second P1 domain to obtain the GST-HB2 construct, and **(v)** attachment of the linker plus a third P1 domain to complete the GST-HB3 construct.

Thus, the cDNA corresponding to the P1 region, RHAMM(518-580), was subcloned into the modified pGEX vector to give GST-HB1, GST-HB2, and GST-HB3 with 1, 2, and 3 repeats of the P1 region, respectively (Figure 2). The sequences of these recombinant constructs were confirmed by DNA sequencing.

2. Example 2: Protein synthesis

Each of the GST-HBM plasmids, as well as the empty pGEX-ERL vector, were transformed into *E. coli* strain BL21 (DE3) (Novagen). Bacteria were grown in 20 ml LB culture at 37 °C overnight, transferred to one liter of fresh LB, and incubated at 37 °C for 3 h. Expression was induced by addition of 0.1 mM IPTG (Pierce) (for GST alone and GST-HB1) or 0.5 mM IPTG (for GST-HB2 and GST-HB3) and incubated at 22 °C for 4 h. The bacterial pellet was collected by centrifugation (4000 × g, 15 min), resuspended with 100 ml of STE buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA), and incubated for 15 min on ice. Next, a mixture of 1 mM each of protease inhibitors (PMSF, aprotinin, pepstatin A, leupeptin, Sigma, St. Louis, MO) and 5 mM dithiothreitol, (DTT, Sigma) were added. The expressed proteins were released into solution by sonication and the 13,000 × g (10 min) supernatant was loaded onto an 10ml total volume of Glutathione-Sepharose 4B bead slurry (equal to 5ml beads, Amersham Pharmacia, Piscataway, NJ) in order to bind GST-tagged proteins. After six washes with PBS (pH 7.4, 0.1 M), the desired proteins (GST, GST-HB1, GST-HB2, and GST-HB3) were eluted with ten bead volumes of 20 mM GSH (Sigma) in Tris-HCl (100 mM, pH 8.0, 120mM NaCl, 0.1% Triton X-100). The elution was repeated two additional times to give three samples for each protein. Protein concentrations were determined by Bradford Reagent (Sigma) with bovine serum albumin (BSA, Pierce) as standard control. Purified proteins were stored at -80°C in small portions. For each use, an aliquot was thawed and discarded after use in a given experimental set. These constructs were first expressed at 37°C. However, the large proportion of the proteins were present in insoluble form; by reducing the expression temperature to 22°C, the percentage of soluble protein was dramatically increased (Figure 3a). Subsequently, GST protein alone and GST-HB1, GST-HB2, and GST-HB3 were purified by affinity chromatography on immobilized

GSH and electrophoresed on SDS-PAGE to show the expected sizes of 25, 30, 38, 46 kDa, respectively (Figure 2b). Protein concentrations decreased as the inserted fragment size increased. Thus, GST and GST-HB1 were obtained at yields of 30 mg per liter bacterial culture, while we initially obtained yields of 10 mg/l for GST-HB2 and 5 mg/l for GST-HB3 The yield of GST-HB3 was increased to 14 mg/l by adding 120 mM NaCl and 0.1% Triton X-100 to the elution buffer. All proteins were relatively stable when maintained at or below -20 °C; binding activity gradually degraded at 4 °C over several months.

3. Example 3: Enzyme-linked immunosorbant assay (ELISA)

For each well in a 96-well plate pre-coated with streptavidin (SA) (Roche, Indianapolis, IN), 50 μl of 10 μg/ml biotinylated heparin (average 15 kDa, Celsus, Cincinnati, OH) was loaded and incubated at 4 °C overnight. Following three washes with TBS (20 mM Tris, 150 mM NaCl, pH 7.5), 100 μl StabilGuard solution (Surmodics, Eden Prairie, MN) was applied to each well to block the unbound SA sites. After 1 h incubation at room temperature (rt), followed by three washes with TBS, triplicate 100 μl aliquots of GST, GST-HB1, GST-HB2 and GST-HB3 were added at increasing concentrations. After 1 h incubation at room temperature, followed by four washes with TBS, 50μl of mouse anti-GST antibody (Sigma) (1:1000 diluted in TBS) was added. After incubation (1 h, rt), the plate was washed four times with TBS. Then, 50 μl horseradish peroxidase (HRP) conjugated anti-mouse IgG (Sigma) (1:3000 diluted in TBS) was added. After incubation (1 h, rt), the plate was washed four times with TBS, and then 100 μl of 3,3',5,5'-tetramethyl benzidene (TMB, Sigma) was added. The wells gradually developed a dark blue color during 15 min incubation. Finally, 100 μl of 1 M H_2SO_4 was added and the resulting yellow color was read by measuring absorbance at 430 nm.

For the competitive ELISA with different GAGs, an aliquot of 100 μl/well of unlabeled GAG was added to the GST or GST-HB proteins (50 μg/ml) after the StabilGuard blocking step but before the antibody loading step. GAGs employed included chondroitin 4-sulfate (CS-A) and chondroitin 6-sulfate (CS-C), keratan sulfate (KS), heparan sulfate (HS) (all from Sigma), HA (190 kDa, produced by acid degradation of 1200 kDa HA from Clear Solutions Biotech, Inc., Stony Brook, NY) and UFH (average 15 kDa, Sigma).

The affinity and selectivity of GST and the GST-HB proteins for HA was examined first, using an ELISA system similar to that described herein but with biotinylated HA as the immobilized ligand. The GST-HB3 protein bound with highest affinity to immobilized HA

and was selective for HA as compared to CS-A and CS-C . Surprisingly, 190 kDa HA was also a poor competitor for displacement of this binding, while 1000-fold lower concentrations of heparin effectively competed for the interaction of GST-HB3 with immobilized HA. Apparently, the tandem repeats of P1 selectively amplified the heparin affinity while reducing the HA affinity. Thus, we repeated the ELISA protocols using biotinylated heparin instead the biotinylated HA. Each of the GST-HB proteins was readily displaced using UFH as the competitor, with a protein concentration of 50 μg/ml (100μl/well) of GST-HB3 (Figure 4).

4. Example 4: Heparin quantification using GST-HB3 protein

The GST-HB3 protein was selected for further heparin measurements using the competitive ELISA. Thus, serial twofold dilutions of UFH were prepared from 10 μg/ml to 20ng/ml, and duplicate aliquots of 100μl/well were used as competitors as described above, with 100 μl ×50 μg/ml aliquot per well of GST-HB3. In addition, 100 μl/well human plasma sample (Sigma) was premixed with 100μl/well of serially diluted heparin and added to plate. In this simulated plasma assay, both UFH and LMWH (6 kD, Sigma) were employed as competitors. Gradient concentrations were also used in this assay to study the feasibility of a role for the GST-HB3 protein in heparin detection in plasma samples.

To evaluate the specificity of GST-HB proteins, a competitive ELISA was performed with CS-A, CS-C, HA, KS, HS, and UFH as the competitors at 200 μg/ml (Figure 5). The results indicated that the GST-HB proteins bound to heparin with higher affinity and selectivity relative to other GAGs. Moreover, both affinity and selectivity appeared to increase with the number of tandem P1 domains. This can be attributed in part to increased electrostatic interactions between the highly-sulfated heparin and HS with the polybasic nature of the binding site. The differences between heparin and HS, which differ little in net charge, can be attributed to stereospecific ligand recognition. Serial dilutions of HA, CS-C, CS-A, and UFH were used with GST and each GST-HB protein. Table 5 presents the estimated IC_{50} values for competitive displacement for each protein, illustrating a 100-2000-fold selectivity for heparin over the less sulfated GAGs. Figure 6 depicts the raw data for GST-HB3.

Table 5. Estimated IC_{50} values (μg/ml) for GAGs as competitors in ELISA with immobilized heparin and GST-HB detection.

GAG	GST-HB1	GST-HB2	GST-HB3
HA	20-50	>200	>200
KS	>1000	>1000	>1000
CS-A	10-20	20-50	20-50
CS-C	100-200	20-50	20-50
Heparin	0.1-0.2	<0.1	0.1-0.2
HS	<1	<5	<5

Example 5 : Quantification of free heparin in solution

GST-HB3 was selected for further study as a detection protein for determination of heparin concentrations. First, serial twofold dilutions of UFH were prepared in the range 10 μg/ml to 20 ng/ml. The UFH sodium salt used was from porcine mucosa. The ELISA data for these dilutions yielded a logarithmic plot of absorbance vs. UFH concentration, and a log-log plot of relative absorbance (corrected for no heparin blank) vs. concentration gave the expected linear relationship (Figure 7). This calibration curve demonstrates that GST-HB3 binding to immobilized biotinylated heparin provides a linear range for detection of free UFH of at least three decades, suggesting that this ELISA has significant potential for measurement of heparin concentrations with high sensitivity as well as high selectivity. The effect of ionic strength was measured by varying the salt concentration from 50 to 1000 mM NaCl. The optimal sensitivity was observed at 150mMNaCl, the physiological concentration employed for this assay. An inverse ELISA, in which immobilized GST-HB3 was coupled to detection by biotinylated heparin and HRP-SA, gave essentially identical results for sensitivity of heparin detection.

Example 6 : Quantification of heparin in human plasma

To determine the suitability of GST-HB3 for determining therapeutic heparin levels in plasma, human plasma was spiked with heparin calibration standards. Aliquots of human

plasma were mixed with equal volumes of serial dilutions prepared from both UFH (average size 15 kDa) and LMWH (average size, 6 kD). The log-log plot of relative absorbance vs. heparin concentration again gave straight lines with the same slope as for the calibration standards in buffer alone (Figure 8). Moreover, both UFH and LMWH showed the same slopes. Essentially, no loss of sensitivity was observed for detection of UFH in serum vs. buffer (dotted line), but as expected, the LMWH was detected with lower sensitivity. The optimal range for heparin measurement appears to be from 10 ng/ml to 20,000 ng/ml for UFH and from 40 ng/ml – 20,000 ng/ml for LMWH. With a parallel experiment performed using Accucolor Heparin Kit (Sigma), this corresponds to 0.01 U/ml to 50 U/ml for UFH and 0.3 U/ml to 2 U/ml for LMWH. Therapeutic levels in plasma are generally between 0.01 and 10.0 units per milliliter, indicating that the assay is sufficiently sensitive to monitor therapeutically relevant changes in heparin levels. The experiments disclosed herein showed the intra-assay coefficient of variance (CV) was <9% for 6 serial UFH dilutions from 78 ng/ml to 2.5 μg/ml, while the inter-assay CV was <12% for three different plasma products obtained from Sigma. Moreover, throughout this detection range, no interference was caused by the presence of up to 5 μg/ml HA in the diluted plasma samples (data not shown). Even 10-fold higher caused minimal interference.

The addition of fresh human plasma did not reduce the absorbance in this ELISA (Figure 14), indicating that human plasma sample itself would not interfere with the competition observed with heparin. That is, no net change in the slopes or intercepts for the linear log-log plots was observed when plasma was added in the assay. However, plasma samples stored at 4 °C for 4 months did affect ELISA absorbance somewhat, suggesting that interfering materials can accumulate in outdated plasma (Figure 14). Ideally, therefore, fresh plasma samples should be used in the assay.

The data suggests that patient variability is minimal, and thus a direct heparin concentration could be read following performance of a generic calibration. This new detection method offers a substantial improvement in the current clinical heparin measurement protocols, as it is faster, more sensitive, more quantitative, and more readily integrated into a hospital clinical chemistry service.

Example 7: Characterization of HB3 binding with heparin

The heparin binding ELISA was performed using different NaCl concentrations in TBS to observe the salt effect. Thus, the HB3 concentration was varied from 0 to 300 μg/ml and

NaCl concentration varied from 150 mM to 1000 mM. After the GST-HB3 was loaded into the wells and incubated with the plate for 1 h, an aliquot from each plate well was transferred into another 96-well plate in spatially corresponding wells. The HB3 contained in those aliquots was considered as unbound and the concentration was measured using the Bradford reagent (Sigma). Next, bound HB3-heparin amount was calculated by Scatchard analysis from the proportional ELISA signal (A_{max}=2.00 in this experiment) at 150 mM NaCl. All heparin added was immobilized in plate, as verified in previous titration with different heparin amounts (data not shown). Thus, the amount of unbound heparin amount equaled to the total heparin (corresponding to the maximum signal) minus bound heparin (corresponding to the measured absorbances). Therefore, the binding K_d value K_d = [unbound HB3][unbound heparin]/[bound HB3-heparin] is considered. Absorbance signals at 300μ μg/ml were selected for K_d calculation because signals at lower concentrations were too weak and variable. Next, the logarithm of K_d value at different NaCl concentrations was plot versus logarithm of [NaCl] to give the number of ionic interactions between HB3 and heparin based on polyelectrolyte theory (PET)[63].

To understand the interactions between GST-HB3 and heparin and the ionic contributions involved, the binding affinity changes were tested as the ionic strength was varied. By increasing NaCl concentrations from 15mM to 1000 mM in TBS, the binding between HB3 and heparin was decreased (Figure 15). By obtaining the concentrations of unbound HB3, unbound heparin and bound HB3-heparin complex, we calculated the K_d value at different NaCl concentrations (Table 6) to quantify the decreased binding with increased ionic strength. It is expected that for most heparin binding proteins, a substantial contribution to binding would arise from the electrostatic interactions between the highly anionic heparin and a correspondingly cationic protein. Increased ionic strength would lessen these ionic interactions between negatively charge sulfate and carboxylate groups on heparin with the positively charged Arg and Lys residues of the protein. For a given heparin binding interaction, an equation based on polyelectrolyte theory (PET) is used to describe such ionic interactions:

$$\log K_d = \log K_d' + Z\Psi \log[Na^+]$$

Here K_d' is the dissociation constant at 1 M [Na^+], the Z value refers to the number of ionic interactions involved in the binding and Ψ is defined as the fraction of Na^+ bound per heparin charge and released upon binding to HB3 (estimated to be ~ 0.8 (32)). Thus by

plotting log K_d vs log [Na^+], we were able to obtain $Z\Psi'\Psi$ value from the slope and the interception, which equals to log K_d', gave us the non-ionic interaction estimation (Figure 16). From the figure Z = 2.50, showing between 2 and 3 ionic interactions per binding heparin - HB3 interaction. Also based on Gilbert equation:

$$\Delta G = -RT(\ln K_d)$$

where R = 8.314 J/(mol·°K) and T = 298 °K. When $K_d = K_d$' at 1 M [NaCl], it is considered as non-ionic interaction and ΔG = 27.1 kJ. Compared with K_d at normal [NaCl] (150 mM), when ΔG = 37.4 kJ, the binding contribution was calculated from non-ionic interactions equals to 27.1/37.4 = 72% and thus the ionic interactions contribute only 28% of the total binding energy. This binding character is in the middle range of known heparin-protein interactions, and acceptable for development of HB3 as a heparin sensor.

Table 6. Kd values at different NaCl concentrations in ELISA with immobilized heparin and GST-HB detection.

[NaCl] (M)	Kd (nM)
0.15	2.7×10^2
0.30	2.2×10^3
0.50	2.6×10^3
0.75	6.1×10^3
1.0	1.8×10^4

Example 8: The Use of Horseradish Peroxidase in Assays

Horseradish peroxidase is used widely in all types of immunoassays as a colorogenic or fluorogenic enzyme. Reaction products can be either soluble or insoluble (precipitates) depending on the substrate used. It has the advantage of being low molecular weight (40,000 g/mol), thereby allowing smaller conjugates, with antibodies, for example, and short reaction times. Furthermore, it has a high specific activity and is highly stable. HRP is extensively used in ELISA as well as other membrane-based assays. With

tetramethylbenzidine (TMB) as a substrate, it is highly sensitivity when coupled to HBMs. One example of using HRP with the ELISAs described herein follows.

HRP was dissolved in 0.1 M NaHCO3, pH 9.5. It was then oxidized with NaIO4, separated via size exclusion, then added in excess to HBM, which had been dialyzed vs. the same buffer. The two proteins were allowed to react via Schiff base formation, then reduced with NaCNBH3. The reaction was then quenched with lysine. The conjugate was then dialyzed vs. PBS, concentrated about 15X, and applied to a size exclusion column to separate HBM-HRP conjugates from unlabeled HRP. Fractions were taken and assayed for HRP activity using TMB substrate solution. Active fractions from the first eluted peak were pooled and concentration of conjugate determined via optical absorbancy. Heparin binding activity was found to reside in the first peak eluting (highest molecular weight), corresponding to HBM-HRP conjugates.

Example 9: Unfractionated Heparin (UFH) ELISA Kit for Plasma Samples

Reagents

In this example, the kit includes Heparin-coated 96-well plates, UFH (Sigma) Standard (170 μg/ml), Detector-Enzyme Conjugate, Conjugate Diluent, TMB Solution, Stop Solution (0.5M H_2SO_4), Wash Concentrate 10X, (diluted 1 part plus 9 parts dH_2O to make TBS-0.05% TWEEN 20), Standard Diluent, [TBS (150 mM NaCl, 10 mM Tris pH 7.5)]. Pipettes, absorbance microplate reader, normal plasma, and a plate cover can also be included.

General

This heparin-ELISA kit is a quantitative enzyme-linked assay designed for the *in vitro* measurement of unfractionated heparin levels in plasma. This assay measures heparin directly using a heparin binding protein which has been conjugated to HRP. The heparin-ELISA is a competitive assay in which the colorimetric signal is inversely proportional to the amount of heparin present in the sample. Samples to be assayed were first mixed with the Detector-Enzyme Conjugate in wells of the heparin coated plate. Heparin in the sample competes with heparin bound to the plate for binding of the Detector-Enzyme Conjugate. The concentration of heparin in the sample was determined using a standard curve of known amounts of heparin. In one embodiment,

the heparin used for the standard curve can match the type of heparin being assayed.

Method of Using Kit

Dilutions of the Heparin Standard were made into normal plasma to obtain standards of 0.03, 0.1, 0.3, 1.0, 3.0 and 10.0 μg/mL. Kit standards were prepared from UFH from Sigma. 9.1mL of conjugate diluent was measured and add to a clean tube. A 'clean transfer' of the lyophilized Detector-Enzyme Conjugate was placed into the 9.1 mL of conjugate diluent. This can be done by adding 500 microliters of the measured Diluent to reconstitute the Detector-Enzyme in the vial. 60 seconds passed to allow the lyophilized material to dissolve and then the liquid was added back to the tube. This step was repeated two more times to be sure all the Detector-Enzyme Conjugate had been transferred from the vial to the tube. Reconstituted Detector-Enzyme Conjugate can be stored at 4°C for no more than 7 days.

A 1:10 dilution of 10X Wash Buffer in distilled or deionized water was made, and a heparin ELISA plate was set up as in Figure 19. The heparin standard dilution series can be run in triplicate. 10 μL of Standards and samples were added into corresponding wells. 90 μL of Working Detector - Enzyme Conjugate was added to all wells except the Blank wells, and then they were mixed well. The plate was covered and incubated for one hour at room temperature. A rotator can be used.

The solution was then discarded and the wells were washed four times with 300 μL per well of 1X Wash Buffer. An automated plate washer can be used. After washing, the next step was immediately carried out, and the wash buffer was immediately removed from the wells. The plate was not allowed to dry. 100 μL TMB solution was added to each well. The plate was incubated in the dark at room temperature for 10-30 minutes waiting for the zero heparin wells to develop to a medium to dark blue color. Color development was watched and overdevelopment not allowed.

50 μL Stop Solution was added which changed the color from blue to yellow. The absorbance of each well was then measured at 450 nm. The binding percentage was calculated for each sample using the formula:

$$[A450(\text{Sample}) - A450(\text{Blank})] / [A450(\text{Zero heparin}) - A450(\text{Blank})] \times 100 = \%\ \text{Binding}$$

Using linear or nonlinear regression, a standard curve of percent binding versus concentration of heparin standards was plotted. Heparin levels of unknowns were determined by comparing their percentage of binding relative to the standard curve. Heparin can be estimated by comparing the values from the wells containing unknowns to the values in the standard curve.

Example 10: Unfractionated Heparin (UFH) ELISA Kit for Buffer/Urine Samples

Reagents

In this example, the kit includes Heparin-coated 96-well plates, UFH (Sigma) Standard (10 µg/ml), Detector-Enzyme Conjugate, Conjugate Diluent, TMB Solution, Stop Solution (0.5M H2SO4), Wash Concentrate 10X, (dilute 1 part plus 9 parts dH2O to make TBS-0.05% TWEEN 20), Standard Diluent, [TBS (150 mM NaCl, 10 mM Tris pH 7.5)]. Pipettes, absorbance microplate reader, and a plate cover can also be included.

General

This heparin-ELISA kit is a quantitative enzyme-linked assay designed for the *in vitro* measurement of unfractionated heparin levels in low protein content fluids such as buffer or urine. This assay measures heparin directly using a heparin binding protein which has been conjugated to HRP. The heparin-ELISA is a competitive assay in which the colorimetric signal is inversely proportional to the amount of heparin present in the sample. Samples to be assayed were first mixed with the Detector-Enzyme Conjugate in wells of the heparin coated plate. Heparin in the sample competes with heparin bound to the plate for binding of the Detector-Enzyme Conjugate. The concentration of heparin in the sample was determined using a standard curve of known amounts of heparin.

Method of Using Kit

Dilutions of the Heparin Standard were made into normal plasma to obtain standards of 0.03, 0.1, 0.3, 1.0, 3.0 and 10.0 µg/mL. Kit standards were prepared from UFH from Sigma. 9.1mL of conjugate diluent was measured and add to a clean tube. A 'clean transfer' of the lyophilized Detector-Enzyme Conjugate was placed into the 9.1 mL of conjugate diluent. This can be done by adding 500 microliters of the measured Diluent to reconstitute the Detector-Enzyme in the vial. 60 seconds passed to allow the lyophilized

material to dissolve and then the liquid was added back to the tube. This step was repeated two more times to be sure all the Detector-Enzyme Conjugate had been transferred from the vial to the tube.

A 1:10 dilution of 10X Wash Buffer in distilled or deionized water was made, and a heparin ELISA plate was set up as in Figure 19. The heparin standard dilution series can be run in triplicate. 10 µL of Standards and samples were added into corresponding wells. 90 µL of Working Detector - Enzyme Conjugate was added to all wells except the Blank wells, and then they were mixed well. The plate was covered and incubated for one hour at room temperature. A rotator can be used.

The solution was then discarded and the wells were washed four times with 300 µL per well of 1X Wash Buffer. An automated plate washer can be used. After washing, the next step was immediately carried out, and the wash buffer was immediately removed from the wells. The plate was not allowed to dry. 100 µL TMB solution was added to each well. The plate was incubated in the dark at room temperature for 10-30 minutes waiting for the zero heparin wells to develop to a medium to dark blue color. Color development was watched and overdevelopment not allowed.

50 µL Stop Solution was added which changed the color from blue to yellow. The absorbance of each well was then measured at 450 nm. The binding percentage was calculated for each sample using the formula:

[A450(Sample) - A450(Blank)]/ [A450(Zero heparin)-A450(Blank)] x 100 = % Binding

Using linear or nonlinear regression, a standard curve of percent binding versus concentration of heparin standards was plotted. Heparin levels of unknowns were determined by comparing their percentage of binding relative to the standard curve. Heparin can be estimated by comparing the values from the wells containing unknowns to the values in the standard curve.

Example 12: Low Molecular Weight Heparin (LMWH) ELISA Kit for Buffer/Urine Samples

Reagents

In this example, the kit includes Heparin-coated 96-well plates, LMWH (Sigma)

Standard (10 μg/ml), Detector-Enzyme Conjugate vial, Conjugate Diluent, TMB Solution, Stop Solution (0.5M H2SO4), Wash Concentrate 10X, (diluted 1 part plus 9 parts dH2O to make TBS-0.05% TWEEN 20), Standard Diluent [(TBS (150 mM NaCl, 10 mM Tris pH 7.5)]. Pipettes, absorbance microplate reader, and a plate cover can also be included.

General

This heparin-ELISA kit is a quantitative enzyme-linked assay designed for the *in vitro* measurement of low molecular weight heparin levels in low protein content fluids such as buffer or urine. This assay measures heparin directly using a heparin binding protein which has been conjugated to HRP. The heparin-ELISA is a competitive assay in which the colorimetric signal is inversely proportional to the amount of heparin present in the sample. Samples to be assayed were first mixed with the Detector-Enzyme Conjugate in wells of the heparin coated plate. Heparin in the sample competes with heparin bound to the plate for binding of the Detector-Enzyme Conjugate. The concentration of heparin in the sample was determined using a standard curve of known amounts of heparin. Heparin used for the standard curve can match the heparin being assayed.

Method of Using Kit

Dilutions of the Heparin Standard were made into normal plasma to obtain standards of 0.03, 0.1, 0.3, 1.0, 3.0 and 10.0 μg/mL. Kit standards were prepared from from Sigma material. 5.2 mL of conjugate diluent was measured and add to a clean tube. A 'clean transfer' of the lyophilized Detector-Enzyme Conjugate was placed into the 5.2 mL of conjugate diluent. This can be done by adding 500 microliters of the measured Diluent to reconstitute the Detector-Enzyme in the vial. 60 seconds passed to allow the lyophilized material to dissolve and then the liquid was added back to the tube. This step was repeated two more times to be sure all the Detector-Enzyme Conjugate had been transferred from the vial to the tube.

A 1:10 dilution of 10X Wash Buffer in distilled or deionized water was made, and a heparin ELISA plate was set up as in Figure 19. The heparin standard dilution series can be run in triplicate. 50 μL of Standards and samples were added into corresponding wells. 50 μL of Working Detector - Enzyme Conjugate was added to all wells except the Blank wells, and then they were mixed well. The plate was covered and incubated for

one hour at room temperature. A rotator can be used.

The solution was then discarded and the wells were washed four times with 300 µL per well of 1X Wash Buffer. An automated plate washer can be used. After washing, the next step was immediately carried out, and the wash buffer was immediately removed from the wells. The plate was not allowed to dry. 100 µL TMB solution was added to each well. The plate was incubated in the dark at room temperature for 40-60 minutes waiting for the zero heparin wells to develop to a medium to dark blue color. Color development was watched and overdevelopment not allowed.

50 µL Stop Solution was added which changed the color from blue to yellow. The absorbance of each well was then measured at 450 nm. The binding percentage was calculated for each sample using the formula:

$$[A450(Sample) - A450(Blank)] / [A450(Zero\ heparin) - A450(Blank)] \times 100 = \%\ Binding$$

Using linear or nonlinear regression, a standard curve of percent binding versus concentration of heparin standards was plotted. Heparin levels of unknowns were determined by comparing their percentage of binding relative to the standard curve. Heparin can be estimated by comparing the values from the wells containing unknowns to the values in the standard curve.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.